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SISTEMAS PURINÉRGICO E COLINÉRGICO E PERFIL OXIDATIVO NO ENCÉFALO DE ROEDORES: INFLUÊNCIA DO ALUMÍNIO E DE DIFERENTES DIETAS

TESE DE DOUTORADO

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Santa Maria, RS, Brasil
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SISTEMAS PURINÉRGICO E COLINÉRGICO E PERFIL OXIDATIVO NO ENCÉFALO DE ROEDORES: INFLUÊNCIA DO ALUMÍNIO E DE DIFERENTES DIETAS

por

Rosilene Rodrigues Kaizer

Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito para a obtenção do grau de **Doutor em Bioquímica Toxicológica**

Orientadora: Prof. **Maria Rosa Chitolina Schetinger**

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SISTEMAS PURINÉRGICO E COLINÉRGICO E PERFIL OXIDATIVO NO ENCÉFALO DE ROEDORES: INFLUÊNCIA DO ALUMÍNIO E DE DIFERENTES DIETAS

elaborada por
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como requisito parcial para obtenção do grau de
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DEDICATÓRIA

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...à memória de minha mãe Ana Lucimar, pela vida, por todo o amor, por sua doação, pelos ensinamentos que me deixaste de herança!

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“Se você quer que as coisas
mudem, você tem que mudar”.
(Jim Rohn)

RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica
Universidade Federal de Santa Maria

SISTEMAS PURINÉRGICO E COLINÉRGICO E PERFIL OXIDATIVO NO ENCÉFALO DE ROEDORES: INFLUÊNCIA DO ALUMÍNIO E DE DIFERENTES DIETAS

AUTORA: ROSILENE RODRIGUES KAIZER
ORIENTADORA: MARIA ROSA CHITOLINA SCHETINGER
Data e local de Defesa: Santa Maria, 05 de março de 2008.

Neste estudo, investigamos os efeitos do alumínio (Al) e de diferentes dietas, individualmente e em associação, através da determinação da atividade das enzimas NTPDase, 5'-nucleotidase e acetilcolinesterase (AChE) no encéfalo de roedores. Adicionalmente, investigamos o estresse oxidativo, através da atividade da enzima antioxidante catalase, e a peroxidação lipídica pela medida dos níveis de TBARS. Ratos machos foram expostos ao Al (50 mg/kg/dia) através de gavagem, por um período de 3 meses. Após o tratamento, foi determinada a atividade das enzimas NTPDase e 5'-nucleotidase em sinaptossoma de córtex cerebral, hipocampo e plaquetas. A hidrólise dos nucleotídeos ATP, ADP e AMP foi aumentada, nas frações sinaptossomais de córtex cerebral e hipocampo bem como nas plaquetas. A atividade da AChE e os níveis de TBARS foram determinados em homogeneizado de diferentes estruturas cerebrais de camundongos expostos ao Al (2,7 mg/kg/dia), através de gavagem, por um período de 3 meses. Quanto à atividade da AChE, o grupo que recebeu Al+citrato de sódio apresentou um aumento da atividade desta enzima em hipocampo, estriado, córtex e hipotálamo. Já o grupo que recebeu só Al apresentou uma diminuição da atividade em hipotálamo e um aumento em estriado. Além disto, a atividade da AChE foi determinada em S1 de diferentes estruturas cerebrais, sinaptossoma de córtex cerebral, e em eritrócitos de ratos machos expostos à Al (50 mg/kg/dia), através de gavagem, por 3 meses. A atividade da AChE apresentou um aumento em S1 de estriado e hipotálamo, e em sinaptossoma de córtex cerebral e eritrócitos. Porém, em sobrenadante (S1) de cerebelo, hipocampo e córtex houve uma diminuição. Adicionalmente, foi determinado o efeito de dietas ricas em gordura saturada e açúcar refinado sobre a atividade da enzima AChE em homogeneizado de diferentes estruturas encefálicas, e atividade da enzima catalase em fígado, e os níveis de TBARS em plasma e fígado de ratos machos e fêmeas. A atividade da

AChE em hipocampo, córtex e hipotálamo de ratos machos e fêmeas foi diminuída, após exposição a ambas as dietas, rica em gordura e rica em açúcar. Nas estruturas cerebelo e estriado não houve alteração na atividade da AChE. Após o consumo de ambas as dietas, a atividade da enzima catalase foi aumentada em fígado de ratos machos e fêmeas. Adicionalmente, considerando todas as alterações ocasionadas pela exposição individual aos fatores ambientais, Al e dietas, foi avaliado o efeito da associação entre esses dois fatores. Dessa forma, após um período de 3 meses de exposição conjunta ao Al (50 mg/kg/dia) através de gavagem e o consumo *ad libitum* de dietas ricas em gordura saturada e gordura saturada/poliinsaturada, foram determinadas a atividade das enzimas NTPDase e 5'-nucleotidase em sinaptossomas de córtex cerebral e plaquetas de ratos. Os animais que receberam ambas as dietas administradas em conjunto com Al e Al/Ci apresentaram um aumento na hidrólise dos nucleotídeos ATP, ADP e AMP, em sinaptossoma de córtex cerebral e plaquetas. Os resultados obtidos no presente estudo relatam que a exposição de roedores a ambos os fatores ambientais Al e dietas ricas em gordura saturada e açúcar refinado, individualmente e em conjunto, afetam os sistemas purinérico e colinérgico, e causam estresse oxidativo.

Palavras-chave: Alumínio (Al), dietas, gordura, açúcar, NTPDase, 5'-nucleotidase, Acetilcolinesterase (AChE), estresse oxidativo, rato, camundongo.

ABSTRACT

Doctoral Dissertation
Post-Graduate Program in Toxicological Biochemistry
Universidade Federal de Santa Maria

THE PURINERGIC AND CHOLINERGIC SYSTEMS AND THE OXIDATIVE PROFILE IN THE BRAIN OF RODENTS: THE INFLUENCE OF ALUMINIUM AND DIET

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Place and date: Santa Maria, March 05, 2008.

In this study, the effects of Aluminium (Al) and of different diets, both individually and in association, were investigated through the determination of NTPDase, 5'-nucleotidase and acetylcholinesterase (AChE) activities in rat brain. In addition, we investigated said effects on oxidative stress by determining activity of the antioxidant enzyme catalase as well as lipid peroxidation by measuring TBARS levels. Male rats were exposed to Al (50 mg/kg/day) by gavage during three months. NTPDase and 5'-nucleotidase activities were then determined in synaptosomes of cerebral cortex and hippocampus, as well as in platelets. ATP, ADP and AMP hydrolysis was increased in both synaptosomes of cerebral cortex and hippocampus, as well as in platelets. AChE activity and TBARS levels were determined in homogenate of different brain structures in mice exposed to Al (2.7 mg/kg/day) by gavage during three months. The group that received Al+sodium citrate presented an increase in AChE activity in hippocampus, striatum, cortex and hypothalamus. On the other hand, the group that received only Al presented a decrease in AChE activity in hypothalamus and an increase in striatum. Moreover, AChE was determined in S1 of different brain structures, synaptosomes of cerebral cortex and erythrocytes of male rats exposed to Al (50 mg/kg/day) by gavage during three months. There was an increase in AChE activity in S1 of striatum and hypothalamus and in synaptosomes of cerebral cortex and erythrocytes. However, in S1 of cerebellum, hippocampus and cortex there was a decrease. In addition, the effect of diets rich in saturated fat and refined sugar on AChE activity in homogenate of different brain structures, on catalase activity in liver and on TBARS levels in plasma and liver were determined in female and male rats. There was a decrease in AChE activity in hippocampus, cortex and hypothalamus of male and female rats given both a diet rich in saturated fat and a diet rich in refined sugar. There was no alteration of AChE activity in cerebellum and striatum. For both diets, catalase

activity was increased in liver of male and female rats. In addition, considering the alterations brought about by the individual exposure to both environmental factors, Al and diets, the effect of the association of both factors was evaluated. Thus, after a period of three months of exposure to both Al (50 mg/kg/day) by gavage and diets rich in saturated fat and saturated/polyunsaturated fat *ad libitum*, NTPDase and 5'-nucleotidase activities were determined in synaptosomes of cerebral cortex and platelets of rats. Animals receiving both diets in association with Al and Al/Ci presented an increase in ATP, ADP and AMP hydrolysis in synaptosomes of cerebral cortex and platelets. The results obtained in the present study demonstrate that exposure to both environmental factors, Al and diets rich in saturated fat and refined sugar, either individually or in association, affected the purinergic and cholinergic systems and caused oxidative stress in rats.

Keywords: Aluminium (Al), diets, fat, sugar, NTPDase, 5'-nucleotidase, Acetylcholinesterase (AChE), oxidative stress, rats, mice.

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LISTA DE ABREVIATURAS

$^{\cdot}OH$ – radical hidroxil

$^{1}O_2$ – oxigênio singleto

ACh – acetilcolina

AChE – acetilcolinesterase

ACR – região conservada da apirase

AD – Doença de Alzheimer

ADP – adenosina 5’-difosfato

Al – alumínio

AMP – adenosina 5’-monofosfato

ATP – adenosina 5’-trifosfato

Aβ – beta amilóide

BBB – barreira hemato-encefálica

BDNF – fator neurotrófico derivado do encéfalo

CH/PL – colesterol/fosfolipídeos

ChAT – colina-acetiltransferase

CHT – transportador de colina

CoA – coenzima A

ColQ – cauda colagênica Q

ERO – espécies reativas de oxigênio

FAO – Organização para a Alimentação e Agricultura

GPI – glicosilfosfatidil inositol

H_2O_2 – peróxido de hidrogênio

LDH – enzima lactato desidrogenase

LDL – lipoproteína de baixa densidade

mAChR – receptor muscarínico da acetilcolina

MDA – malondialdeído

nAChR – receptor nicotínico da acetilcolina

NTPDase – ecto-nucleosídeo trifosfato difosfoidrolase

$O^{\cdot -}$ - ânion superóxido

O_2 – oxigênio

OMS – Organização Mundial de Saúde

P1 – receptor da adenosina

P2X – receptor ionotrópico de nucleotídeos

P2Y – receptor metabotrópico de nucleotídeos

PAS – sítio aniônico periférico

PUFA – ácidos graxos poliinsaturados

SNC – sistema nervoso central

SNP – sistema nervoso periférico

TBARS – espécies reativas ao ácido tiobarbitúrico

TM1 – terminal amino

TM2 – terminal carboxi

UDP – uridina 5’-difosfato

UTP – uridina 5’-trifosfato

vAChT – transportador vesicular-acetilcolina

SUMÁRIO

1. INTRODUÇÃO

Atualmente, o estilo de vida e o ambiente desempenham um papel fundamental na manutenção da função neural dos indivíduos, e estes fatores podem estar relacionados com a etiologia de diversas desordens neurológicas. A Revolução Industrial gerou um grande progresso às sociedades ocidentais, porém a disponibilidade de metais que ocorrem naturalmente no ambiente, foi aumentada significativamente. Como foi o caso do uso extensivo do alumínio (Al) na indústria e na agricultura, onde a acidificação do solo leva ao aumento da disponibilidade do Al (LUKIW & MCLACHLAN, 1995).

O Al é um dos metais mais abundantes e representa cerca de 8% da crosta terrestre, apesar disso, não apresenta função biológica conhecida (SUWALSKY et al., 2004). Os mecanismos celulares da neurotoxicidade do Al estão relacionados com os riscos ambientais e ocupacionais. As fontes potenciais de exposição ao Al incluem alimentos, medicamentos, como antiácidos e antidiarréicos, vacinas, água potável, utensílios de cozinha, cosméticos e desodorantes (YOKEL & MCNAMARA, 2001). O Al é conhecidamente um elemento neurotóxico (YOKEL, 2000). A neurotoxicidade do Al se deve a sua presença no encéfalo, a qual é permitida devido à capacidade deste metal em influenciar a permeabilidade da barreira hemato-encefálica (BBB), alterando o fluxo de moléculas e íons dentro e fora do encéfalo (BANKS et al., 1988). Dessa forma, há o reconhecimento do Al como um agente neurotóxico, e vários estudos relatam seu envolvimento na etiologia da Doença de Alzheimer (AD) (BONDY et al., 1998; EXLEY & KORCHAZHKINA, 2001; GONÇALVES & SILVA, 2007).

Outro fator relacionado ao progresso das sociedades ocidentais é o consumo de dietas ricas em açúcar-refinado e gordura saturada. Estudos relatam que essas dietas contribuem para o declínio cognitivo no envelhecimento (KNOPMAN et al., 2001) e aceleram o curso da demência na AD (KALMIJN et al., 1997, 2000). Realmente, a gordura saturada presente nas dietas causa alterações na composição lipídica das membranas plasmáticas, afetando a atividade de enzimas ligadas à mesma. Em adição, alterações na razão molar de colesterol/fosfolipídeos (CH/PL) em nível de biomembranas, podem levar a uma condição de vulnerabilidade à intoxicação ao Al (SILVA et al., 2002). Assim, o Al pode afetar a atividade de enzimas ligadas à membrana, como a NTPDase e 5'-nucleotidase e AChE.

O ATP é uma das principais moléculas extracelulares sinalizadoras em todo o corpo (ABBRACCHIO & BURNSTOCK, 1998). As ectonucleotidases NTPDase (E.C. 3.6.1.5) e 5'-nucleotidase (E.C. 3.1.3.5) participam no controle dos níveis de ATP na fenda sináptica e atuam no controle da neuromodulação e neurotransmissão purinérgica (SCHETINGER et al.,

2001; BALZ et al., 2003). A enzima NTPDase hidrolisa os nucleotídeos extracelulares di e trifosfatados na presença de Ca^{2+} ou Mg^{2+}, sendo bem caracterizada no sistema nervoso central (SNC), em plaquetas e outros tecidos (PILLA et al., 1996; SCHETINGER et al., 2001; BALZ et al., 2003). Estudos sugerem um papel para a NTPDase no controle da neurotransmissão, e alterações na atividade desta enzima parecem estar associadas com diferentes processos relacionados ao aprendizado e aquisição de memória (ZIMMERMANN, 2001). Adicionalmente, a enzima 5'-nucleotidase catalisa a hidrólise do AMP, liberando como produto a adenosina, um importante neuromodulador (ZIMMERMANN, 2001). O Al pode interferir na neurotransmissão purinérgica, devido à característica do ATP de se ligar avidamente ao Al em pH fisiológico, formando o complexo Al-ATP. Essa ligação é mais constante do que a sua habitual ligação ao co-fator, magnésio (MACDONALD & MARTIN, 1988). Além disso, o alumínio pode alterar a estrutura lipídica de membranas celulares, afetando o transporte de íons e conseqüentemente alterando a atividade de enzimas dependentes de íons, como a NTPDase e 5'-nucleotidase dependem de cátions divalentes (SUWALSKY et al., 2000). Dessa forma, alterações na estrutura das membranas plasmáticas, ocasionadas pelo Al ou devido a fatores da dieta, como o colesterol, podem levar a mudanças na atividade destas enzimas.

A neurotransmissão é um processo dinâmico, sustentado por um ciclo permanente de liberação de neurotransmissores, havendo a liberação de mais de um neurotransmissor em resposta à estimulação. Dessa forma, o ATP é conjuntamente liberado com o neurotransmissor acetilcolina, que é também inativado enzimaticamente na fenda sináptica, pela acetilcolinesterase (GONÇALVES & SILVA, 2007). A acetilcolinesterase (AChE, E.C. 3.1.1.7) é uma importante enzima regulatória, que hidrolisa rapidamente o neurotransmissor acetilcolina (ACh), encontrada principalmente no encéfalo, músculos, eritrócitos e neurônios colinérgicos (MESULAM et al., 2001)**.** A atividade da AChE é sensível a fatores exógenos que incluem dietas (RUANO et al., 2000; OLIVIER et al., 2001; VAJRESWARI et al., 2002) e exposição a metais como o Al (ZATTA et al., 2002a). Além disso, sabe-se que o Al interage com o sistema colinérgico, agindo como uma colinotoxina (GULYA et al., 1990). Os efeitos estimulatórios e inibitórios de ensaios *in vivo* e *in vitro* do alumínio na atividade da AChE têm sido descritos na literatura (PATOCKA, 1971; YATES et al., 1980; MARQUIS et al., 1984; KOWAL et al., 1989; GULYA et al., 1990; PENG et al., 1992; ZATTA et al., 1993; KUMAR, 1998; PLATT et al., 2001; ZATTA et al., 2002a; YOUSEF, 2004). Esses diversos efeitos obtidos podem ser explicados por diferenças nos métodos e doses de administração do Al, diferenças nas amostras biológicas, diferentes períodos de exposição e diferença na

especiação do metal. Ao mesmo tempo, acredita-se que fatores das dietas também afetam a atividade das colinesterases (VAJRESWARI et al., 2002; RUANO et al., 2000; OLIVIER et al., 2001; MCGEHEE et al., 2000). As alterações na atividade da AChE causadas por diferentes dietas podem ocasionar mudanças nos fosfolipídeos e cadeias de ácidos graxos prejudicando a degradação da ACh. As dietas também podem alterar a disponibilidade de precursores para a síntese de ACh, ou alterar a atividade da própria enzima.

Vale lembrar a possível geração de estresse oxidativo nas células de animais expostos a fatores oxidantes, como o Al e a diferentes componentes da dieta. Há muitos relatos indicando que o Al interage com as membranas celulares, que constituem seu principal alvo, induzindo alterações estruturais e funcionais das membranas, gerando a peroxidação lipídica (JONES & KOCHIAN, 1997; TAKANO & SHIMMEN, 1999; ZATTA & SUWALSKY, 2001). O SNC é particularmente vulnerável às espécies reativas de oxigênio (EROS) devido à alta concentração de lipídeos nas membranas celulares neuronais (ZATTA et al., 2002b)

Considerando a possibilidade do envolvimento de fatores ambientais como Al e dietas ricas em gordura saturada e açúcar refinado, na etiologia de diversas desordens neurológicas e ao mesmo tempo, o fato dos neurotransmissores ATP e ACh serem co-liberados na fenda sináptica, o intuito do presente trabalho foi investigar alterações nos sistemas purinérgico e colinérgico e perfil oxidativo de roedores expostos ao Al e às dietas individualmente, bem como a administração conjunta de ambos.

1.1 Objetivos

1.2 Objetivo Geral

Avaliar o efeito da exposição ao alumínio por um longo período, em baixas e altas doses, bem como o consumo de diferentes dietas, e a associação de ambos nos sistemas colinérgico e purinérgico.

1.3 Objetivos Específicos

- Verificar alterações na atividade da enzima AChE (E.C. 3.1.1.7) em diferentes estruturas encefálicas de camundongos machos expostos à baixa concentração de alumínio.

- Determinar alterações na atividade das ecto-enzimas NTPDase (E.C. 3.6.1.5) e 5'-nucleotidase (E.C. 3.1.3.5) em sinaptossomas de córtex cerebral e hipocampo e plaquetas de ratos expostos ao alumínio.

- Verificar alterações na atividade da enzima AChE entre sinaptossoma de córtex cerebral, S1 de diferentes estruturas encefálicas e eritrócitos de ratos expostos à alta concentração de Al.

- Verificar alterações na atividade da enzima AChE em diferentes estruturas encefálicas de ratos machos e fêmeas submetidos a diferentes dietas.

- Verificar os efeitos do alumínio e das dietas sobre o estresse oxidativo através da medida do TBARS e da atividade da enzima catalase em diferentes tecidos de ratos expostos ao alumínio e diferentes dietas.

- Determinar o efeito da associação entre o Al e dietas ricas em gordura saturada sobre a atividade das ecto-enzimas NTPDase e 5'- nucleotidase em sinaptossomas de córtex cerebral e plaquetas de ratos.

2. REVISÃO DE LITERATURA

2.1 Alumínio

Os humanos são freqüentemente expostos ao Al devido ao fato deste metal ser o terceiro elemento mais abundante na crosta terrestre (EXLEY, 2003). O isolamento do Alumínio (Al), o 13º elemento da tabela periódica, em 1827 é atribuído a Wohler (1827 apud GONÇALVES & SILVA, 2007). Porém, apesar desta grande abundância em sua distribuição no ambiente o Al não apresenta uma função biológica conhecida (SUWALSKY et al., 2004). O Al é um metal altamente citotóxico para plantas e animais, e é responsável por perdas significativas na produção agrícola mundial e uma série de desordens neurológicas em animais e humanos. O desenvolvimento industrial e o uso de fertilizantes têm contribuído para a acidificação das áreas agrícolas e habitats aquáticos (HAUG, 1984; TAYLOR, 1991). Esta acidificação do ambiente aumenta a biodisponibilidade do Al (LUKIW & MCLACHLAN, 1995). Atualmente, o alumínio é amplamente utilizado e as suas ligas e compostos são cruciais em muitos setores industriais (GONÇALVES & SILVA, 2007), levando à exposição ocupacional de indivíduos. O alumínio é um constituinte de aditivos de alimentos e medicamentos, tais como antiácidos, antidiarréicos, aspirinas, além de cosméticos, desodorantes e também é encontrado em utensílios de cozinha (YOKEL, 2000). Sais de Al são frequentemente utilizados na água como floculantes para remover a cor e a turbidez, durante os processos de purificação e tratamento da água (FLATEN, 2001). Estas são as rotas primárias de exposição ao alumínio para a maioria dos seres humanos (SUTHERLAND & GREGER, 1998). Dessa forma, cada organismo desde plantas e animais apresenta pequenas quantidades de Al (SCHETINGER et al., 1999; WILLIAMS, 1999; YOKEL, 2002), que pode ser encontrado praticamente em todos os tecidos de mamíferos como o encéfalo, fígado, rins, coração, sangue e ossos (DOMINGO et al., 1996; YOKEL et al., 1996; SCHETINGER et al., 1999). Vale salientar que a presença do Al no encéfalo se deve a sua capacidade em atravessar a barreira hemato-encefálica (BBB) (YOKEL, 2002) onde se acumula nas células nervosas e gliais, chegando a alcançar concentrações micromolares nas células (GUY et al., 1990; DE STASIO et al., 1994; SUAREZ-FERNANDEZ et al., 1996; LEVESQUE et al., 2000; AREMU & MESHITSUKA, 2005; NAGASAWA et al., 2006). Dessa forma, o Al pode interferir no metabolismo de neurotransmissores e nas atividades de enzimas específicas responsáveis pela síntese e degradação dos mesmos (GONÇALVES & SILVA, 2007).

O Al tem sido reconhecido como um elemento neurotóxico há mais de um século (ZATTA et al., 2002a). Os primeiros estudos com animais que demonstraram a

neurotoxicidade do Al, foram conduzidos por Siem e Dölken (DÖLKEN, 1897; ALFREY, 1993). Em 1965, foi relatado que a inoculação intracerebral de fosfato de Al em coelhos resulta na degeneração neurofibrilar (TERRY & PEÑA, 1965), estas alterações mostraram-se muito semelhantes aos emaranhados neurofibrilares observados na Doença de Alzheimer (AD) (FLATEN, 2001). Desde então surgiram vários debates quanto ao papel do Al na AD. Porém, somente no ano de 1973, foi publicado o primeiro relato do aumento das concentrações de Al no encéfalo de pacientes com AD (CRAPPER et al., 1973).

Ao mesmo tempo, logo após a introdução da rotina de diálise, em pacientes com insuficiência renal, Alfrey e colaboradores descreveram a encefalopatia de diálise (ALFREY et al., 1972; 1976 apud ZATTA et AL., 2003). Esta seria considerada a primeira condição humana relacionada com a exposição ao Al, e talvez a primeira doença iatrogênica reconhecida na população de pacientes que realizam diálise (ALFREY, 1993). Porém, é preciso salientar as diferenças neuropatológicas entre a AD e a encefalopatia de diálise. Na doença de Alzheimer a acumulação do Al ocorre em emaranhados neurofibrilares e placas senis, enquanto na encefalopatia de diálise a acumulação do metal ocorre em todo o encéfalo (ZATTA et al., 2003).

Em 1986, a primeira tentativa de relacionar os níveis reais de Al presentes na água potável com a AD, foi relatada em dois estudos paralelos realizados na Noruega, onde verificou-se que a mortalidade de indivíduos com demência, foi maior em áreas com altas concentrações de Al na água potável (FLATEN, 1986; VOGT, 1986). Mais tarde, estudos epidemiológicos confirmaram a implicação do acúmulo anormal e possível absorção do Al com a AD (MARTYN et al., 1989; FRATAGLIONI, 1998; EXLEY, 2001). Atualmente, a acumulação do Al tem sido associada com uma grande variedade de patologias humanas tais como, anemia, osteodistrofia, encefalopatias, fraqueza muscular e doenças neurodegenerativas, como a AD (GÓMEZ et al., 1998; NICHOLAS et al., 1999; ZATTA et al., 2002a). Realmente, a exposição por um longo período ao Al em ratos pode resultar em uma condição neuropatológica na qual há perda neuronal seletiva e os prejuízos da função colinérgica são evidentes (BILKEI-GORZO, 1993). O efeito neurotóxico do Al pode ser explicado por mecanismos adicionais, como a formação e acumulação da Aβ (beta amilóide) (NAYAK, 2002)**.** De fato, as placas senis e os emaranhados neurofibrilares, as principais lesões da AD, são abundantes em regiões enriquecidas por sinapses colinérgicas (MORÁN et al., 1993; GEULA & MESULAM, 1999).

Sabe-se que um dos sítios primários de toxicidade do Al pode estar relacionado com a sua inserção nos domínios de ligação de metais das enzimas e lipídeos, causando uma

alteração no metabolismo e sinalização celular (MARTIN, 1992; SHI et al., 1993). O Al tem um efeito negativo sobre inúmeras reações bioquímicas importantes envolvendo a ligação de outros metais, como o Mg^{2+} e Ca^{2+} com proteínas (YOKEL et al., 2003). Um exemplo são as enzimas dependentes desses íons tais como a NTPDase e a 5'-nucleotidase, que atuam em cascata, finalizando a hidrólise seqüencial do ATP a adenosina, nas sinapses purinérgicas (SUWALSKY et al., 2000). Além disso, tem sido proposto que o Al pode potencializar as atividades de neurotransmissores através da ação do complexo Al-ATP em receptores de ATP no encéfalo (EXLEY, 1999).

Ao mesmo tempo, estudos demonstram que a interação do Al com fosfolipídeos carregados negativamente causam alterações nas propriedades físicas das membranas (VERSTRAETEN & OTEIZA, 2002). Adicionalmente, devemos considerar que as membranas celulares são fontes ricas de ácidos graxos poliinsaturados o que as torna particularmente vulneráveis ao ataque das EROs (espécies reativas de oxigênio) (PARIHAR et al., 2008). O estresse oxidativo é caracterizado por um significante aumento na concentração de EROs e espécies reativas de nitrogênio, e/ou um decréscimo nos mecanismos de detoxificação celular (SHRADER & FAHIMI, 2006). O Al é considerado um agente pró-oxidante (NAYAK, 2002). O sistema nervoso central (SNC) é particularmente vulnerável a ERO, devido a alta concentração de lipídeos nas membranas celulares neuronais (ZATTA et al., 2002b). O estresse oxidativo gerado está envolvido com várias patologias humanas, como por exemplo, a ateroesclerose, a injúria de reperfusão na isquemia e hipertensão (CORRÊA et al., 2007), no câncer (MALDONADO et al., 2006), no diabetes tipo 2, ou em doenças neurodegenerativas tais como Mal de Parkinson e AD (SHRADER & FAHIMI, 2006). Realmente, o Al pode ocasionar efeitos neurotóxicos, por produzir mudanças na estrutura e na função da membrana plasmática (JULKA & GILL, 1996), podendo conseqüentemente alterar a atividade de enzimas ligadas à mesma.

A co-ingestão do Al com ligantes pode reciprocamente influenciar a absorção gastrointestinal de ambos. Alguns ligantes na dieta demonstram um aumento na absorção do Al, o citrato de sódio tem importante papel no mecanismo de transporte do Al (BERTHON, 2002). O citrato atua como veículo, onde a passagem do Al depende de sua ligação a um citrato livre (EXLEY, 1999). O complexo Al-citrato é formado pela ligação do Al com o grupo hidroxil e dois terminais carboxilados do citrato (GREGOR & POWELL, 1986). (Figura 1)

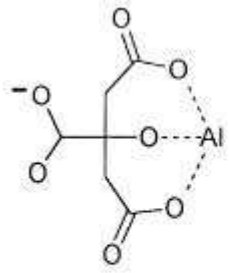

FIGURA 1 – A estrutura do complexo Al.Citrato. Adaptado de Yokel e colaboradores (1999).

A resposta biológica à exposição a um composto químico pode ser bifásica. A resposta bifásica é caracterizada por um aumento na resposta a dose baixa, e uma diminuição em resposta a uma dose mais alta (KUMAR, 1999). O Al é capaz de produzir um efeito bifásico em diversos sistemas celulares. Por exemplo, uma alta dose de Al pode diminuir a síntese de proteínas enquanto que uma dose baixa pode aumentar a síntese de proteínas (KUMAR, 1999). De fato, vários trabalhos de exposição *in vitro* e *in vivo* ao Al relatam o efeito bifásico nas enzimas colinérgicas (PATOCKA, 1971; YATES et al., 1980; MARQUIS & BLACK, 1984; GULYA et al., 1990; EXLEY, 1999).

2.2 Dietas

A obesidade nos Estados Unidos (EUA) tem aumentado substancialmente nos últimos anos (FLEGAL et al., 1998; MUST et al., 1999; MOKDAD et al., 1999), e atingiu níveis de epidemia (ROBERTS et al., 2002). No Brasil, cerca de 38 milhões de brasileiros com mais de 20 anos estão acima do peso. Desse total, mais de 10 milhões são considerados obesos, de acordo com os padrões estabelecidos pela Organização Mundial de Saúde (OMS) e pela Organização para a Alimentação e Agricultura (FAO). Segundo pesquisas o excesso de peso dos brasileiros está relacionado ao aumento do consumo de alimentos industrializados e também pela ingestão de grande quantidade de açúcar e gordura (MINISTÉRIO DA SAÚDE, 2005).

A obesidade está associada com uma variedade de doenças crônicas, incluindo a doença coronariana, a hipertensão, o diabetes melito e certos tipos de câncer (JUNG, 1997; MUST et al., 1999). A etiologia da obesidade é multifatorial, onde predisposições genéticas interagem como estilo de vida do indivíduo, principalmente padrões de dieta e ausência de atividade física. A obesidade é o simples resultado de hiperfagia ou desequilíbrio na razão entre ingestão energética/gasto energético (ROBERTS et al., 2002). No entanto, há indícios

de que a obesidade possa ser induzida sem uma ingestão energética significativamente excessiva. Diversos estudos em modelos animais e em seres humanos têm demonstrado que o aumento dos níveis de carboidratos refinados (sacarose) e/ou gordura saturada nas dietas pode levar à obesidade na ausência de ingestão excessiva de energia (BARNARD et al., 1998a, 1998b; ROBERTS et al., 2002).

Uma vez que a genética dos humanos e outros animais não sofreu grandes alterações ao longo dos últimos 40 anos, o aumento da obesidade parece ser devido ao estilo de vida dos indivíduos, especificamente dieta inapropriada e sedentarismo (ROBERTS et al., 2002). Dessa forma, o estilo de vida é um fator crucial na etiologia de doenças crônicas e na manutenção da função neural, relacionadas à obesidade e outros distúrbios alimentares. Em particular, o consumo de dietas ocidentalizadas, desenvolvidas após a revolução industrial, as quais são ricas em gordura saturada e açúcar refinado (BLOCK et al., 1988; GOLDBART et al., 2006). Um estudo recente em pré-adolescentes com elevados níveis de LDL-colesterol, relatou que o consumo de lanches rápidos, sobremesas e pizzas perfazem cerca de um terço do consumo total de energia dessas crianças (VAN HORN et al., 2005). Realmente, as dietas ocidentais geralmente incluem pelo menos 30% a 40% de energia na forma de gordura, tendo como fonte de lipídeos ácidos graxos saturados e poliinsaturados (SCHRAUWEN & WESTERTERP, 2000). O consumo diário de alimentos per capita nos Estados Unidos apresentou um aumento ao longo dos últimos 40 anos, aumentando de cerca de 3.100 kcal/dia em 1965 para um consumo de 3.900 kcal/dia em 2000 (GAESSER, 2007) (Figura 2).

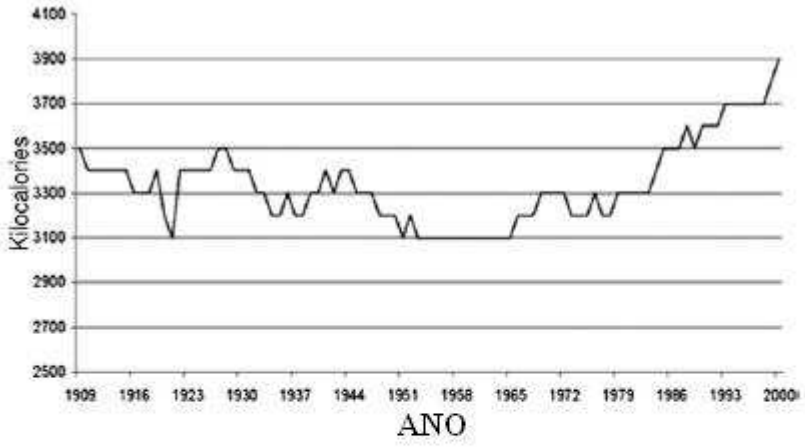

FIGURA 2 – Aumento do consumo diário de calorias nos Estados Unidos, per capita, por dia do ano de 1909 a 2000. Adaptado de Gerrior e colaboradores (2004).

Essas dietas podem contribuir para o declínio cognitivo no envelhecimento (KNOPMAN et al., 2001) e podem acelerar o curso da demência na AD (KALMIJN et al., 1997; 2000). Estudos relatam um número de alterações neuropatológicas em animais expostos

a dietas ricas em gordura saturada, similares aquelas caracteristicamente associadas à AD (SPARKS et al., 2000). A adição de colesterol à dieta resulta no aumento da imunoreatividade à proteína β-amilóide (Aβ) dentro dos neurônios do córtex e hipocampo de animais (XUE et al., 2007). Foi observado em vários estudos a ocorrência de depósitos anormais de placas senis compreendendo a proteína β-amilóide e emaranhados neurofibrilares (GLENNER, 1988; SELKOE, 1992; SOTO et al., 1994). Na AD são observados níveis reduzidos de neurotransmissores e uma extensa perda neuronal e sináptica (SMITH et al., 1995; NEWHOUSE et al., 1997). Especificamente, é relatado um prejuízo da função colinérgica, que está relacionado a um decréscimo na liberação do neurotransmissor ACh em córtex e hipocampo (RAKONCZAY et al., 2005).

Os fatores da dieta também podem afetar a plasticidade neuronal que é a capacidade crítica para compensar desafios, envolvendo mecanismos celulares e moleculares de formação e função de sinapses, crescimento de neuritos e adaptação comportamental (MOLTENI et al., 2002). Assim, Molteni e colaboradores (2002), forneceram evidências de que as dietas ricas em gordura saturada e açúcar refinado podem diminuir a plasticidade neuronal pela via de regulação do fator neurotrófico derivado do encéfalo (BDNF). O BDNF é um mediador crucial da função e vitalidade neuronal, e desempenha um importante papel nos eventos neuronais de aprendizado e memória (CASTRÉN et al., 1998, GOLDBART et al., 2006).

2.3 NTPDase e 5'-nucleotidase

O ATP (adenosina 5'- trifosfato) é uma reconhecida fonte de energia intracelular, um nucleotídeo de purina encontrado em virtualmente todas as células (FIELDS & BURNSTOCK, 2006). Além de seu papel bem estabelecido no metabolismo celular, o ATP extracelular e os produtos de sua hidrólise, ADP e adenosina, apresentam pronunciados efeitos em uma variedade de processos biológicos, incluindo a neurotransmissão, a contração muscular, a função cardíaca e plaquetária, a vasodilatação e o metabolismo do glicogênio hepático (AGTERESCH et al., 1999). Realmente, a sinalização de nucleotídeos extracelulares é reconhecida há quase 20 anos, como um dos mais importantes mecanismos de sinalização intercelular (LUTHJE, 1989; BURNSTOCK & KNIGHT, 2004). Dessa forma, o ATP é reconhecido como um neurotransmissor e neuromodulador no sistema nervoso central (BURNSTOCK & WILLIAMS, 2000; CUNHA & RIBEIRO, 2000; FIELDS & BURNSTOCK, 2006).

Essencialmente, em todas as células de mamíferos há processos de liberação e remoção dos neurotransmissores da fenda sináptica, cada um dos produtos de hidrólise do ATP pode ativar diferentes tipos de receptores para nucleotídeos (ROBSON et al., 2006). Por exemplo, os receptores P2 ligam ATP e ADP, e os receptores P1 ligam adenosina (FIELDS & BURNSTOCK, 2006) (Figura 3). Os receptores P2 se dividem em dois subtipos, os sete subtipos de receptores ionotrópicos (P2X) e oito subtipos de receptores metabotrópicos (P2Y) (ROBSON et al., 2006). Os receptores P2X respondem ao ATP, enquanto que os receptores P2Y podem ser ativados por ATP, ADP, UTP, sendo sensíveis também a pirimidinas, além de nucleotídeos de açúcares, como UDP-glicose e UDP-galactose (NORTH, 2002; LAZAROWSKI, 2003; ABBRACCHIO et al., 2003). Lembrando que os receptores de purinas e pirimidinas são amplamente distribuídos não só no sistema nervoso, como também em muitas células não-neuronais (BURNSTOCK & KNIGHT, 2004). Dessa forma, dependendo do subtipo de receptor P2 e da via de sinalização envolvida, esses receptores podem desencadear e mediar processos de curto prazo (agudo) que afetam o metabolismo celular, neurotransmissão, neuromodulação, secreção endócrina e exócrina, agregação plaquetária, vasodilatação. Além disso, a sinalização purinérgica também provoca profundo impacto sobre outras respostas mais prolongadas, incluindo proliferação celular, diferenciação e apoptose, levando ao envelhecimento, ao câncer e doenças neurodegenerativas (HARDEN et al., 1997; WEISMAN et al., 1998; BURNSTOCK & KNIGHT, 2004).

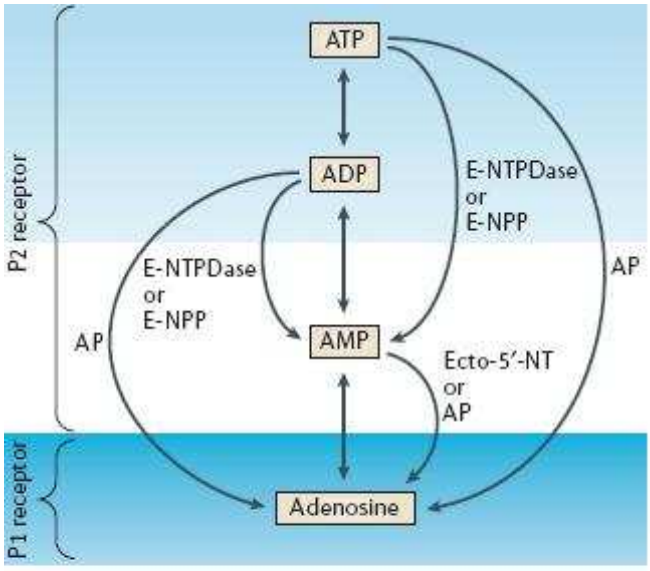

FIGURA 3 – Receptores purinérgicos ligam o ATP extracelular e os produtos das reações que resultam da hidrólise enzimática do ATP por ectonucleotidases. Adaptado de Fields e Burnstock (2006).

Estudos relatam a atividade de membros de uma família de nucleosídeo trifosfato difosfoidrolases como as NTPDase 1 a 8 (E.C. 3.6.1.5), e uma 5'-nucleotidase (E.C. 3.1.3.5,

CD73) podem participar no controle dos níveis extracelulares do ATP na fenda sináptica e no controle da neuromodulação e da neurotransmissão purinérgica (ROBSON et al., 2006). A NTPDase é uma enzima associada à membrana que hidrolisa nucleosídeos extracelulares tri e difosfatos na presença de Ca^{2+} ou Mg^{2+} (ZIMMERMANN et al., 1998; SCHETINGER et al., 1998; ROBSON et al., 2006) e tem sido bem caracterizada no SNC e em outros tecidos, como em plaquetas e em linfócitos (PILLA et al., 1996; ZIMMERMANN, 2001; SCHETINGER et al., 2001; BALZ et al., 2003; LUNKES et al., 2004; LEAL et al., 2005). Recentemente, a NTPDase em vertebrados foi caracterizada em oito formas denominadas NTPDase-1 a NTPDase-8 (ROBSON et al., 2006; GRINTHAL & GUIDOTTI; 2006). Dos oito membros desta família, todos tem dois domínios transmembrana, com exceção das NTPDases 5 e 6 (GRINTHAL & GUIDOTTI, 2006). Além disso, vale salientar, que quatro das NTPDases são enzimas localizadas na superfície celular, com o sítio catalítico voltado para o meio extracelular, são as NTPDase1, 2, 3 e 8 (ROBSON et al., 2006). Como tal, essa família de NTPDases, tem modulado alguns processos de sinalização ou biossintéticos nos quais os nucleotídeos extracelulares desempenham um papel, incluindo a homeostase vascular, a manutenção do tamanho celular, a sinalização celular, a função imune e a modificação de proteínas e lipídeos (BRAKE & JULIUS, 1996; BURNSTOCK, 1998; GAYLE et al., 1998; ENJYOJI et al., 1999; SCHWEIBERT e ZSEMBERY, 2003; MARCUS et al., 2003).

Os subtipos individuais das NTPDases realizam uma variedade de tarefas na célula, exibindo diferentes localizações e especificidades: algumas residem na membrana plasmática, outras no complexo de Golgi, lisossomos, ou retículo endoplasmático (ZIMMERMANN, 2000). Além disso, cada NTPDase tem uma hierarquia característica das preferências para substratos, cátions divalentes e formação de produto (WANG & GUIDOTTI, 1998; ZIMMERMANN, 2001; ROBSON et al., 2006). Realmente, é possível observar essas preferências por substrato, como por exemplo, a NTPDase1 hidrolisa igualmente bem ATP e ADP, diferindo da NTPDase-2 que hidrolisa preferencialmente o ATP (BALZ et al., 2003). As NTPDase3 e 8, por sua vez, revelam uma preferência maior pelo substrato ATP em relação ao ADP (ROBSON et al., 2006). Ao contrário das NTPDase1 e NTPDase2, as NTPDase3 e NTPDase8 são preferencialmente ativadas pelo Ca^{2+}, em relação ao Mg^{2+} (BIGONNESSE et al., 2004; LAVOIE et al., 2004; VORHOFF et al., 2005). Presumivelmente, as diferenças entre os subtipos nas propriedades catalíticas, podem ser devidas a diferenças na seqüência de aminoácidos, como também nas estruturas secundária, terciária e quaternária (GRINTHAL & GUIDOTTI, 2004) (Figura 4).

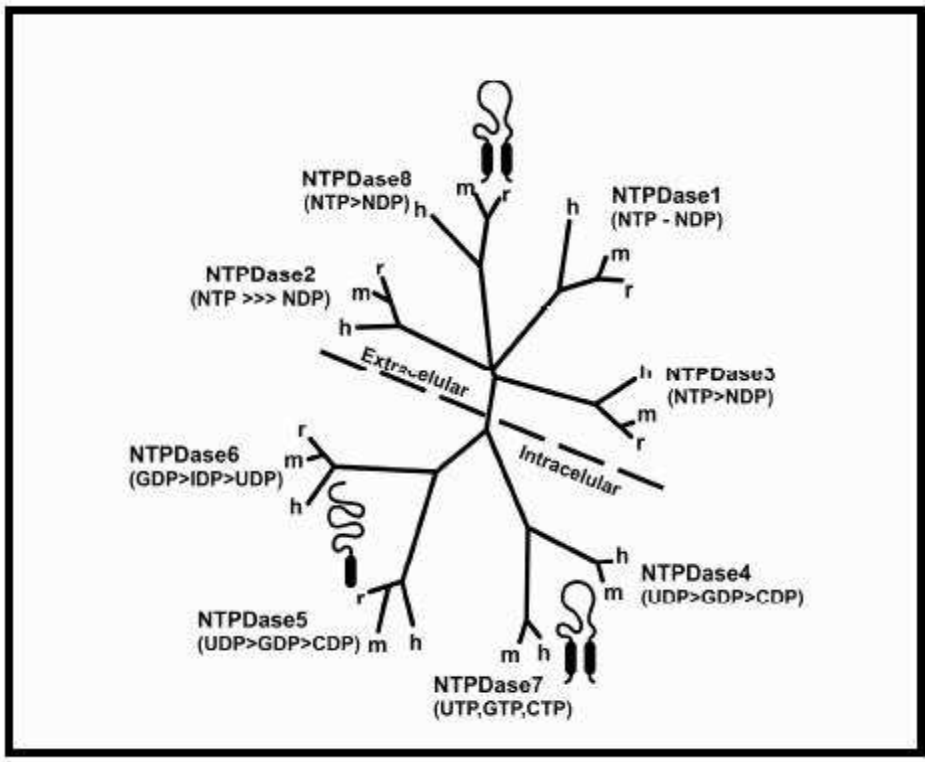

FIGURA 4 – Membros da família de ectonucleotidases, sua localização celular, extracelular ou intracelular, preferência por substrato, além da topografia de cada grupo de enzimas quanto aos domínios transmembrana (1 ou 2). Adaptado de Vorhoff e colaboradores (2005).

Outro aspecto estrutural compartilhado por todos os membros da família das NTPDases é um conjunto de 5 domínios altamente conservados chamados regiões conservadas da apirase (ACRs), denominadas ACR1 a ACR5, as quais tornaram-se uma característica marcante destas enzimas, provavelmente desempenhando um importante papel na formação do sítio catalítico das enzimas (SCHULTE et al., 1999). As NTPDase1, 2, 3 e 8 são firmemente ancoradas à membrana via dois domínios transmembrana, o terminal amino (TM1) e o terminal carboxi (TM2), que como por exemplo, na NTPDase1 são importantes para a manutenção da atividade catalítica e especificidade ao substrato (SCHULTE et al., 1999; GRINTHAL & GUIDOTTI, 2006). Realmente, estudos determinaram que a atividade da CD39 requer a presença de ambos os terminais TM1 e TM2 na membrana (GRINTHAL & GUIDOTTI, 2006).

A 5’-nucleotidase em associação à NTPDase, completa a hidrólise dos nucleotídeos, com a hidrólise do AMP até a produção de adenosina (ZIMMERMANN, 1992). A enzima Ecto-5’-nucleotidase é uma glicoproteína ancorada a glicosilfosfatidil inositol (GPI), e ocorre essencialmente em todos os tecidos, sendo transitoriamente expressa na superfície de células nervosas em desenvolvimento, e nas sinapses durante seu desenvolvimento e remodelação (ZIMMERMANN et al., 1998). A adenosina no SNC é uma importante molécula

neuromoduladora (LOPES et al., 2002), além de atuar como um modulador do tônus vascular e como um inibidor da agregação plaquetária (MENDONÇA et al., 2000; KAWASHINA et al., 2000). A ecto-5'-nucleotidase é ancorada à membrana por um único domínio transmembrana (SEMENZA, 1986). A família de receptores de adenosina P1 é compreendida por A_1, A_2, A_{2B} e A_3, todos acoplados a proteínas G (FIELDS & BURNSTOCK, 2006) (Figura 5).

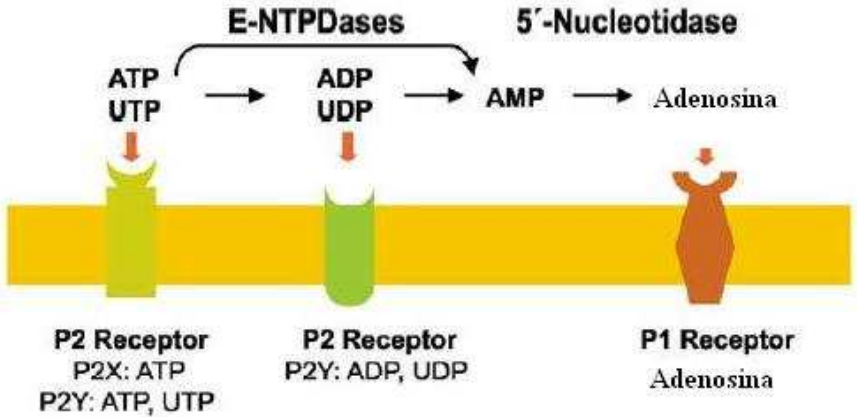

FIGURA 5 – Catabolismo de nucleotídeos extracelulares na superfície celular e ativação potencial de receptores de nucleotídeos (receptores P2) e de adenosina (receptores P1). Adaptado de Kukulski e colaboradores (2005).

2.4 Acetilcolinesterase (AChE)

Os principais componentes que regulam a neurotransmissão colinérgica são: a acetilcolina (ACh), colina-acetiltransferase (ChAT), o transportador de colina (CHT), o transportador de acetilcolina vesicular (vAChT), os receptores muscarínicos (mAChR), receptores nicotínicos (nAChR) e a acetilcolinesterase (AChE) (SARTER & PARIKH, 2005).

A ACh foi o primeiro composto a ser identificado como um neurotransmissor nas sinapses do SNC (VAN DER ZEE & LUITEN, 1999). A ACh desempenha um papel fundamental no SNC e está relacionada ao comportamento, bem como aprendizado e memória, além de atuar na organização cortical do movimento, e controle do fluxo sanguíneo cerebral (MESULAM et al., 2002; MORETTO et al., 2004). No neurônio pré-sináptico a ACh é sintetizada pela enzima colina-acetiltransferase (ChAT) a partir dos precursores acetil-coenzima A (CoA), que doa o grupo acetil, e colina (SOREQ & SEIDMAN, 2001). A acetil-CoA é um produto do metabolismo oxidativo na mitocôndria, e a colina é derivada do metabolismo lipídico ou obtida por recaptação na fenda sináptica (VAN DER ZEE & LUITEN, 1999; SARTER & PARIKH, 2005). Os níveis normais de colina no encéfalo e plasma parecem ser relativamente estáveis em 5-10 μM (LOCKMAN & ALLEN, 2002). Porém, dietas com deficiência e ricas em colina podem reduzir e aumentar, respectivamente,

os níveis plasmáticos de colina, e conseqüentemente, podem também afetar os níveis cerebrais de colina (WURTMAN et al., 2000). Essas alterações no conteúdo de colina podem, por sua vez, afetar a síntese de ACh. Após sua síntese, a ACh é armazenada em vesículas sinápticas via um transportador vesicular-ACh (vAChT), e permanece no terminal pré-sináptico, até que um potencial de ação chegue ao terminal, liberando-a na fenda sináptica (SOREQ & SEIDMAN, 2001) (Figura 6).

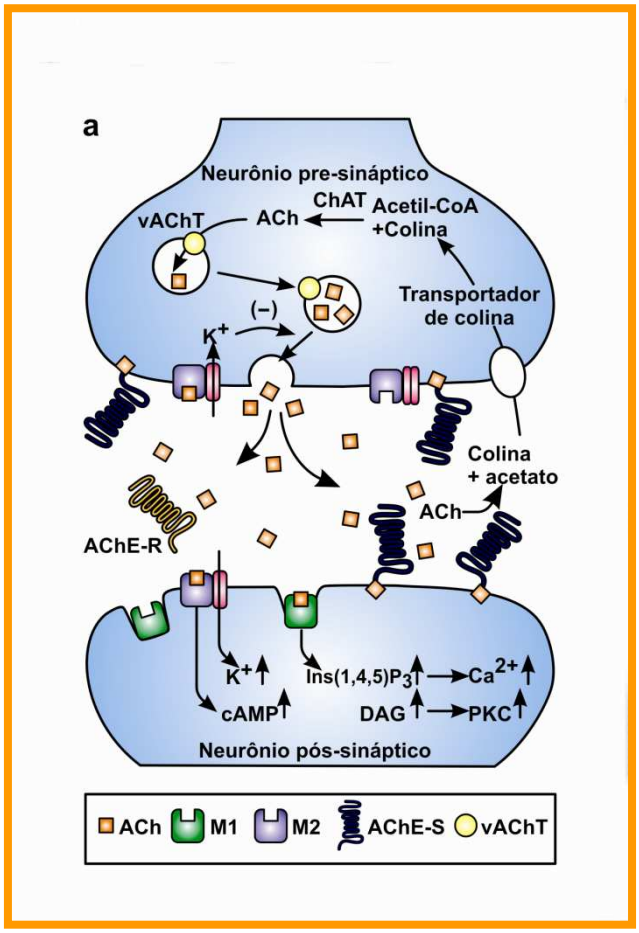

FIGURA 6 – Sinapse colinérgica. Adaptado de Soreq e Seidman, 2001.

A ação da ACh é mediada por receptores muscarínicos e nicotínicos, localizados nas membranas pré e pós-sinápticas transmitindo informação, através da indução de potenciais de ação (SOREQ & SEIDMAN, 2001). Os receptores muscarínicos (mAChRs) são ligados à proteína G, e cinco subtipos foram identificados (M1-M5), sendo que os receptores M1 e M2 estão presentes em neurônios do SNC e sistema nervoso periférico (SNP) (VAN DER ZEE & LUITEN, 1999). Os receptores nicotínicos (nAChRs) atuam como canais iônicos regulados por um ligante, com alta permeabilidade ao Ca^{2+}, e são constituídos por cinco subunidades denominadas α1, α2, β, γ e δ (ARIAS, 2000; DÍAZ-HERNÁNDEZ et al., 2002). Os receptores nicotínicos estão localizados na membrana pós-sináptica, no SNC e nos músculos,

nas junções neuromusculares (ARIAS, 2000). No terminal pré-sináptico, a acetilcolina e o ATP são co-estocados e co-liberados, e ambos apresentam a capacidade de ativar receptores ionotrópicos (WONNACOTT, 1997, MACDERMOTT et al., 1999; KHAKH & HENDERSON, 2000; GONÇALVES & SILVA, 2007). Os receptores ionotrópicos de nucleotídeo, P2X, são abundantemente expressos nos sistemas nervoso central e periférico, e assim como o nAChR, também apresentam alta permeabilidade aos íons Ca^{2+} (KRISHTAL et al., 1988; COLLO et al., 1996; EVANS et al., 1996, CUNHA & RIBEIRO, 2000). Os receptores P2 são muito mais abundantes nos terminais colinérgicos, do que na população total dos terminais sinápticos (DÍAZ-HERNÁNDEZ et al., 2002).

Após efetuar sua função, a ACh é hidrolisada na fenda sináptica pela enzima acetilcolinesterase (AChE, E.C. 3.1.1.7), liberando ácido acético e colina (SOREQ & SEIDMAN, 2001). A colina, por sua vez, é em sua maioria captada por um transportador de colina (CHT) para o terminal pré-sináptico, onde atua como precursora na síntese de uma nova molécula de ACh (SARTER & PARIKH, 2005). A AChE é uma importante enzima regulatória que hidrolisa rapidamente o neurotransmissor ACh nas sinapses colinérgicas, bem como nas junções neuromusculares (GRISARU et al., 1999). A AChE é uma enzima heterogênea encontrada em encéfalo, músculos, eritrócitos e neurônios colinérgicos (MESULAM et al., 2001). A AChE apresenta diferentes formas moleculares, três formas globulares (G1, G2 e G4) e três formas assimétricas (A4, A8 e A12). O SNC contêm, principalmente, as formas globulares, enquanto as formas assimétricas são encontradas principalmente no sistema nervoso periférico (SNP) e músculo (RAKONCZAY et al., 2005). As formas globulares podem ser solúveis ou ancoradas à membrana por seqüências de aminoácidos hidrofóbicos e são preferencialmente expressas no SNC, enquanto outras formas são ligadas à membrana através de glicofosfolipídeos, sendo encontradas nos sistema nervoso, músculo, eritrócitos e linfócitos (TALESA, 2001). As formas assimétricas estão incluídas na matriz extracelular por uma cauda colagênica (ColQ), e estão localizadas nas junções neuromusculares (TALESA, 2001; ALDUNATE et al., 2004) (Figura 7).

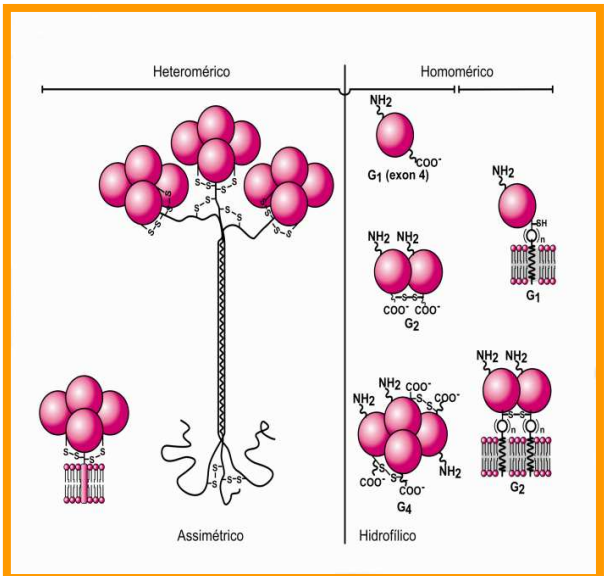

FIGURA 7 – Isoformas da enzima AChE. Adaptado de http://www.chemistry.emory.edu/ach_inactivation.htm.

A AChE mostra algumas características não encontradas em alguma outra enzima, tais como, a organização do sítio ativo e o mecanismo catalítico. O sítio ativo da AChE é encontrado no interior de uma garganta estreita (*gorge*), e consiste de dois subsítios de ligação, um carregado negativamente ou sítio aniônico, e um sítio esterásico, contendo os resíduos catalíticos, também chamados de tríade catalítica (SHAFFERMAN et al., 1992; TALESA, 2001). A tríade catalítica é composta por três resíduos de aminoácidos, serina, histidina e um resíduo ácido, glutamato (SOREQ & SEIDMAN, 2001). Além do sítio catalítico, a AChE apresenta um sítio aniônico periférico (PAS), que está localizado na entrada do sítio ativo *gorge*, este é o sítio de ligação para inibidores e ativadores alostéricos (TAYLOR & LAPPI, 1975). A ACh é hidrolisada a partir de sua ligação ao resíduo de serina no sítio ativo da enzima, formando o intermediário acetil-AChE, liberando colina. Em seqüência, há a hidrólise desse intermediário liberando acetato, e permitindo o "*turnover*" da enzima (SOREQ & SEIDMAN, 2001) (Figura 8).

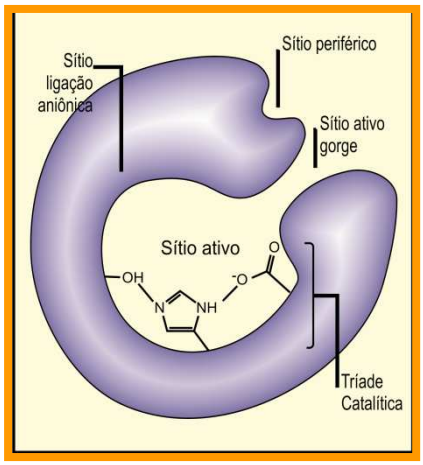

FIGURA 8 - Sítio esterásico contendo a tríade catalítica, externamente o sítio periférico aniônico (PAS) Adaptado de Soreq & Seidman (2001).

Além das propriedades catalíticas a AChE tem um potente efeito na adesão celular (JOHNSON & MOORE, 1999), na extensão dos neuritos (LAYER et al., 1993 apud SOREQ & SEIDMAN, 2001), como também participa na regulação estrutural da diferenciação pós-sináptica (SOREQ & SEIDMAN, 2001). Além disso, a AChE pode estar presente tanto em veneno de cobra quanto no SNC de mamíferos (AHMED et al., 2006) e vários fatores tais como dietas (RUANO et al., 2000; MC GEHEE et al., 2000; OLIVIER et al., 2001) e metais (ZATTA et al., 2002a) podem afetar a sua atividade. Adicionalmente, também é um alvo de neurotoxinas presentes em inseticidas e agentes de guerra química, além de ser um alvo terapêutico no tratamento da depressão e da Doença de Alzheimer (AD) (MESULAM et al., 2001; RACCHI et al., 2004).

Estudos têm relatado a abundância de placas senis e emaranhados neurofibrilares, que são as principais lesões fisiológicas da AD, nas regiões enriquecidas com sinapses colinérgicas (MORÁN et al., 1993; GEULA & MESULAM, 1999). De fato, evidências de que a enzima pode aumentar a agregação da proteína Aβ (ALVAREZ et al., 1997; De FERRARI et al., 2001) e promover a geração de agregados amilóides por acelerar o acúmulo do peptídeo Aβ (INESTROSA et al., 1996; BARTOLI et al., 2003), sugerem o envolvimento da AChE na gênese da AD.

2.5 Estresse oxidativo e defesa antioxidante

Sob condições fisiológicas normais, espécies reativas ao oxigênio (ERO), são formadas em baixas concentrações nas células e tecidos, por meio da redução do oxigênio molecular (GUTTERIDGE & HALLIWELL, 1989; SIES, 1993). O químico francês

Lavoisier foi o primeiro a explicar em 1777 que a combustão e respiração são processos ligados a reações envolvendo oxigênio (ZATTA et al., 2002b). Realmente, o oxigênio (O_2) é a molécula oxidante mais importante nos organismos aeróbicos (PARIHAR et al., 2008). Muitos tipos de EROs são formados dentro dos organismos, as formas mais comuns de EROs incluem: ânion superóxido ($O_2^{\cdot-}$), radical hidroxil ($^{\cdot}OH$), oxigênio "singleto" ($^1O_2$), e peróxido de hidrogênio (H_2O_2). Essas EROs, são produtos do recebimento de 1-elétron do O_2, que é o precursor da maioria das EROs e o mediador nas reações oxidativas em cadeia. O radical hidroxil ($^{\cdot}OH$) é o radical mais reativo, o peróxido de hidrogênio (H_2O_2) é produzido *in vivo* em muitas reações e pode ser convertido no altamente reativo ($^{\cdot}OH$).

Formação de EROs:

$O_2 + e^- \rightarrow O_2^{-}\cdot$

$O_2^{-}\cdot + O_2^{-}\cdot + 2H^+ \rightarrow H_2O_2 + O_2$ $\quad (H_2O_2 \rightarrow 2\ H_2O + O_2)$

$O_2^{-}\cdot + H_2O_2 \rightarrow O_2 + OH\cdot + OH^-$

Peroxidação Lipídica

$ROOH + metal^{n+} \rightarrow ROO\cdot + metal^{n-1} + H^+$

$X\cdot + RH \rightarrow R\cdot + XH$

$R\cdot + O_2 \rightarrow ROO\cdot$

$ROO\cdot + RH \rightarrow ROOH + R\cdot$

$ROO\cdot + ROO\cdot \rightarrow ROOR + O_2$

$ROO\cdot + R\cdot \rightarrow ROOR$

$R\cdot + R\cdot \rightarrow RR\cdot$

As células são capazes de se defender dos efeitos danosos das EROs em condições fisiológicas normais, por meio de mecanismos antioxidantes endógenos que incluem um sistema enzimático e não-enzimático, como vitaminas e algumas moléculas antioxidantes (GUEMOURI et al., 1991). Normalmente, há um equilíbrio entre a produção e a destruição das EROs. Porém, quando este equilíbrio é destruído, as EROs são produzidas excessivamente e todos os tecidos estão expostos ao estresse oxidativo (AUST et al., 1986). Em muitos tecidos a produção aumentada de EROs é promovida pela exposição a diversos fatores exógenos, como o Al que é um agente pró-oxidante (EXLEY, 2004). A peroxidação

lipídica de membranas ocorre quando há um aumento na produção de EROs e/ou uma diminuição nos mecanismos de defesa antioxidante.

A peroxidação lipídica é relatada como uma importante causa do dano oxidativo às membranas celulares, e que eventualmente levam à morte celular (HORTON, 2003). Um bom indicador da injúria oxidativa e um produto final da peroxidação lipídica é a formação de malondialdeído (MDA). Vários estudos têm demonstrado que níveis elevados de MDA estão associados à injúria a tecidos. As membranas plasmáticas são formadas por camadas de moléculas lipídicas, com duplas ligações de ácidos graxos insaturados altamente reativos (ZATTA et al., 2002b). De fato, as membranas celulares são ricas em ácidos graxos poliinsaturados (PUFA) os quais são o alvo mais vulnerável ao ataque das EROs. O cérebro é o alvo preferencial para os processos peroxidativos devido a seu alto conteúdo de ácidos graxos peroxidáveis (PATRICÓ & DELANTY, 2000).

Os organismos vivos têm um complexo sistema antioxidante, enzimático e não-enzimático que protege contra os efeitos deletérios dos radicais livres (SODHI et al., 2008). Os antioxidantes não-enzimáticos são geralmente moléculas pequenas, tais como ascorbato e glutationa, que ocorrem em fases aquosas (fluido intracelular), enquanto os antioxidantes lipofílicos (tocoferóis e carotenóides) são ativos nas membranas celulares (NOCTOR & FOYER, 1998; SMIRNOFF & WHEELER, 2000). Dentre os antioxidantes enzimáticos, estão a superóxido dismutase (SOD; E.C. 1.15.1.1) que desempenha um importante papel nos mecanismos de defesa antioxidante das células contra o estresse oxidativo, convertendo o ânion superóxido à molécula menos reativa H_2O_2 e moléculas de oxigênio (PARIHAR et al., 2008); além da catalase (CAT, E.C. 1.11.1.6) que é uma clássica enzima, da família das oxirredutases, presente nos peroxissomos, que converte H_2O_2 em H_2O (OSHINO et al., 1973). A catalase tem uma importante função protetora contra os efeitos tóxicos dos peróxidos gerados nos peroxissomos e os remove com grande eficiência (SIRAKI et al., 2002).

Além disso, sabe-se que depósitos de Aβ são neurotóxicos *in vivo* e podem estar envolvidos na patogênese da AD (TSAI et al., 2004). Estudos relatam que depósitos conjuntos de ferro e Aβ são fontes significativas de EROs. Em adição, vários outros metais são encontrados associados com placas senis (BEAUCHEMIN & KISILEVSKY, 1998), como é o caso do Al (FLATEN, 2001; ZATTA et al., 2002b) e pode influenciar o depósito de Aβ (HOUSE et al., 2004) e seu potencial para catalisar a formação de EROs (BONDY et al., 1998; YANG et al., 1999).

3. RESULTADOS E DISCUSSÃO

Os resultados e discussão deste trabalho será apresentados em três artigos científicos e dois manuscritos, distribuídos em cinco capítulos, como segue.

Capítulo I: Ativação da acetilcolinesterase e aumento da peroxidação lipídica após longo período de exposição a baixos níveis de alumínio em diferentes regiões encefálicas de camundongos.

Artigo I: Acetylcholinesterase activation and enhanced lipid peroxidation after long-term exposure to low levels of aluminum on different mouse brain regions.

Capítulo II: O efeito do alumínio sobre a atividade da NTPDase e 5'-nucleotidase em sinaptossomas e plaquetas de ratos.

Artigo II: The effect of aluminium on NTPDase and 5'-nucleotidase activities from rat synaptosomes and platelets.

Capítulo III: Efeito da exposição por um longo período ao alumínio sobre a atividade da acetilcolinesterase no sistema nervoso central e eritrócitos.

Manuscrito I: Effect of long-term exposure to aluminium on the acetylcholinesterase activity in the central nervous system and erythrocytes.

Capítulo IV: Alterações induzidas pela dieta na atividade da AChE após longo período de exposição.

Artigo III: Diet-induced changes in AChE activity after long-term exposure.

Capítulo V: Efeito da exposição por um longo período ao alumínio e dieta rica em gordura na atividade da NTPDase e 5'-nucleotidase em sinaptossomas e plaquetas de ratos.

Manuscrito II: Effect of long-term exposure to aluminium and high fat diet on NTPDase and 5'-nucleotidase activities from synaptosomes and platelets of rats.

CAPÍTULO I

ARTIGO I:

Acetylcholinesterase activation and enhanced lipid peroxidation after long-term exposure to low levels of aluminum on different mouse brain regions

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Acetylcholinesterase activation and enhanced lipid peroxidation after long-term exposure to low levels of aluminum on different mouse brain regions

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Abstract

Aluminum (Al), oxidative stress and impaired cholinergic functions have all been related to Alzheimer's disease (AD). The present study evaluates the effect of aluminum on acetylcholinesterase (AChE) and lipid peroxidation in the mouse brain. Mice were loaded by gavage with Al 0.1 mmol/kg/day 5 days per week during 12 weeks. The mice were divided into four groups: (1) control; (2) 10 mg/mL of citrate solution; (3) 0.1 mmol/kg of Al solution; (4) 0.1 mmol/kg of Al plus 10 mg/mL of citrate solution. AChE activity was determined in the hippocampus, striatum, cortex, hypothalamus and cerebellum and lipid peroxidation was determined in the hippocampus, striatum and cortex. An increase of AChE activity was observed in the fourth group (Al + Ci) in the hippocampus (36%), striatum (54%), cortex (44%) and hypothalamus (22%) ($p < 0.01$). The third group (Al) presented a decrease of AChE activity in the hypothalamus (20%) and an enhancement in the striatum (27%). Lipid peroxidation, measured by TBARS (thiobarbituric acid reactive substances), was elevated in the hippocampus and cerebral cortex when compared with the control ($p < 0.01$). The effect of aluminum on AChE activity may be due to a direct neurotoxic effect of the metal or perhaps a disarrangement of the plasmatic membrane caused by increased lipid peroxidation.

Keywords: Acetylcholinesterase; AChE; Aluminum; Lipid peroxidation

1. Introduction

Several factors augment the risk of AD, including environmental factors, such as metals. However, the exact role of metals in Alzheimer's etiology remains unclear and controversial. Evidence that Al contributes to AD remains contradictory, however, epidemiological studies have indicated a link between increased Al concentration in potable water and AD [1,2]. Similarly, a variety of human and animal studies have implicated learning and memory deficits after Al exposure [3].

The precise molecular mechanism by which Al exerts its neurotoxic effects is still not completely understood. However, literature data suggest that Al interacts with the cholinergic system, acting as a cholinotoxin [4]. The intensification of inflammations and the interference with cholinergic projection functions may represent the way by which Al contributes to pathological processes in AD leading to learning and memory deficits [5].

In vitro and in vivo effects of Al on acetylcholinesterase (AChE; E.C. 3.1.1.7) activity have been described in a large number of studies [4,6–17], yet results are contro-

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versial and both activation and inhibition of AChE activity have been reported. In fact, these controversies could be explained by the different methods and doses of Al administration, differences in the biological samples assayed, differing periods of exposure (long-term and short-term) and by the metal speciation.

The brain may be particularly vulnerable to oxidative damage and it is known that one of the pathways of neuronal damage and death in AD is mediated by free radical injury, mainly because the brain is rich in peroxidizable fatty acids [18]. In respect to lipid peroxidation, Al is not a transition metal and does not undergo redox reactions in vivo. However, increased reactive oxygen species have been reported during aluminum exposure [19]. In fact, Al might very well exert its toxic effects by interfering with pathways involved in normal iron metabolism and homeostasis [20,21], being considered a pro-oxidant agent [22].

Besides the fact that aluminum is a cholinotoxin and a pro-oxidant agent, its neurotoxic effects could be exerted by additional mechanisms such as the promotion and accumulation of insoluble amyloid β protein, the aggregation of hyperphosphorylated tau protein, disruption of calcium regulation and by interacting directly with the genomic structure [21].

Yokel and McNamara [23] have suggested that low level long-term exposure to Al may be a contributing factor in AD and related disorders. Based on this and on the knowledge that in AD there are alterations in the cholinergic system and there is also oxidative injury, we performed our experiment using a low oral dose of Al by gavage during three months (60 administrations) and measuring both AChE activity and lipid peroxidation in different brain regions such as the hippocampus, cerebral cortex, striatum, hypothalamus and cerebellum.

2. Materials and methods

2.1. Animals

Adult male mice (*Swiss albino*), aged 7–8 weeks, weighing 30–45 g obtained from our breeding colony were used. Animals were maintained on a 12:12 light/dark cycle, in an air-conditioned temperature (22 ± 1 °C) colony room, with free access to water and commercial chow (Supra, Brazil). All the procedures were approved by the Institutional Commission from the Federal University of Santa Maria and are in agreement with the International Council.

2.2. Materials

Acetylthiocholine iodide, 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB), sodium dodecylsulfate (SDS), malondialdehyde (MDA), thiobarbituric acid and trizma base were purchased from Sigma Chemical Co (St. Louis, MO). All other reagents used in the experiments were of the highest purity available.

2.3. Treatment

The animals were subjected to gavage for five consecutive days followed by two days of no treatment each week, completing a total of 60 administrations (12 weeks). Gavage was performed using a syringe with a modified steel point to introduce the solution into the mouse's esophagus without injuring the tissue. Mice were divided into four groups: (1) control animals, that received only ultrapure water ($n = 6$); (2) animals treated with 10 mg/kg of citrate solution ($n = 6$); (3) animals treated with Al 0.1 mmol/kg diluted in ultrapure water ($n = 6$); (4) animals treated with Al 0.1 mmol/kg plus 10 mg/mL of citrate solution ($n = 6$). The total volume of the solution per treatment per day by gavage was approximately 40–50 μL.

The dose utilized in this study (0.1 mmol Al/kg) was considered low when compared with other studies using oral Al administration [14–16,24–26]. Citrate was used because it increases Al absorption [27,28], probably by enhancing Al solubility [22].

The animals were euthanized 24 h after the last dose and the brains were dissected and collected immediately in beakers and maintained on ice (5 °C).

2.4. AChE activity assay

The brain was excised rapidly and the structures were isolated (hippocampus, striatum, cerebellum, cortex and hypothalamus). The brain regions were then homogenized in a glass potter in the same solution of 320 mM sucrose, 0.1 mM EDTA, 5 mM Tris–HCl, pH 7.5, at a proportion of 1:10 (g/v).

AChE activity was determined according to Ellman et al. [29], modified by Rocha et al. [30]. The reaction mixture (2 mL final volume) was composed of 100 mM phosphate buffer pH (7.5), 1 mM 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB). The method is based on the formation of a yellow anion, 4,4′-dithio-bis-acid nitrobenzoic measured by absorbance at 412 nm during 3-min incubation at 25 °C. The enzyme (40–50 μg of protein) was pre-incubated for 2 min. The reaction was initiated by adding 0.8 mM acetylthiocholine iodide. The enzyme activity was expressed in μmoles AcSCh/h/mg of protein.

2.5. Brain TBARS measurement

Brain TBARS levels were determined by the method described previously by Ohkawa et al. [31] with a few

modifications [32]. In short, the reaction mixture contained 200 μL of brain homogenates or standard (MDA-malondialdehyde 0.05 mM), 200 μL of 8.1% sodium dodecylsulfate (SDS), 750 μL of acetic acid solution (2.5 M HCl, pH 3.5) and 750 μL of 0.8% TBA. Since sucrose interferes in the TBARS assay, a portion of the brain was weighed, homogenized at a proportion of 1 g of tissue to 10 mL of buffer Tris/HCl 10 mM pH 7.4 plus 10% of sodium dodecylsulfate (SDS) 10%. The mixtures were heated at 95 °C for 90 min. After centrifugation at 1700*g* for 5 min, the absorbance was measured at 532 nm. TBARS tissue levels were expressed as nmol MDA/per mg of protein.

2.6. Protein determination

Protein was measured by the Coomassie blue method according to Bradford [33] using bovine serum albumin as standard.

2.7. Statistical analysis

Data were analyzed by analysis of variance (One-way ANOVA) followed by the Tukey–Kramer multiple comparisons test. All analyses were performed using the GraphPad InStat software.

3. Results

AChE activity was modified by Al in the hippocampus ($p < 0.01$) and post hoc comparisons by Tukey–Kramer's test revealed that the enzyme activity was significantly higher in the group that received Al + Ci (Fig. 1). There were similar results for AChE activity in the cerebral cortex (Fig. 2). In the striatum AChE activity was enhanced in the presence of Al ($p < 0.01$), in all Al groups. Post hoc comparisons by Tukey–Kramer's multiple range test revealed that the Al and Al + Ci groups presented a significant elevation of acetylthiocholine hydrolysis in comparison to the control and Ci groups (Fig. 3). In the hypothalamus, the effect of Al on AChE depended on the presence of citrate ($p < 0.01$). Post hoc comparisons by Tukey–Kramer's test revealed that AChE activity was significantly higher in the Al + Ci group and significantly lower in the Al group when compared to the control and Ci groups (Fig. 4). In the cerebellum there was no alteration in enzyme activity in the presence of Al (Fig. 5).

TBARS production was altered in the hippocampus and post hoc comparisons by Tukey–Kramer's multiple range test revealed that the Ci, Al and Al + Ci groups were different from the control group (Fig. 6A). In the cortex the Ci, Al and Al + Ci groups were different from the control group ($p < 0.01$) (Fig. 6B). In the striatum there was no difference in TBARS production among the groups (Fig. 6C).

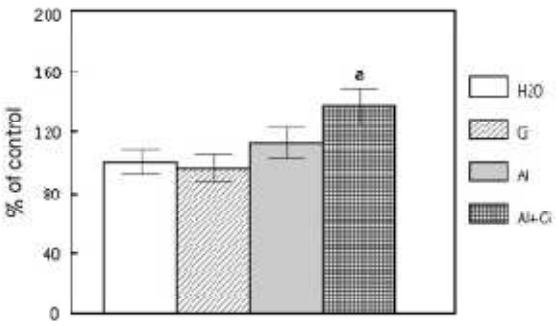

Fig. 1. Effect of oral Al administration on AChE activity in the hippocampus of adult rats. Each column represents mean ± SD ($n = 6$) as percent of control. AChE control value was 3.29 ± 0.1 and was expressed as μmol AcSCh/h/mg of protein. [a] Different from the others when $p < 0.01$ (ANOVA – Tukey–Kramer's test).

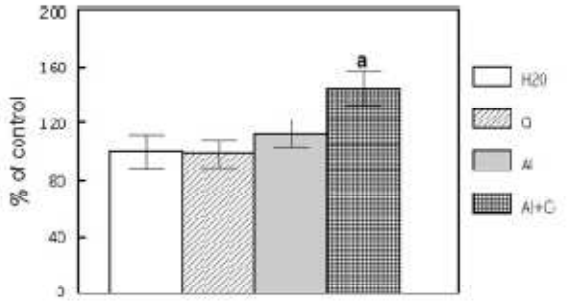

Fig. 2. Effect of oral Al administration on AChE activity in the cortex of adult rats. Each column represents mean ± SD ($n = 6$) as percent of control. AChE control value was 5.94 ± 0.8 and was expressed as μmol AcSCh/h/mg of protein. [a] Different from the others when $p < 0.01$ (ANOVA – Tukey–Kramer's test).

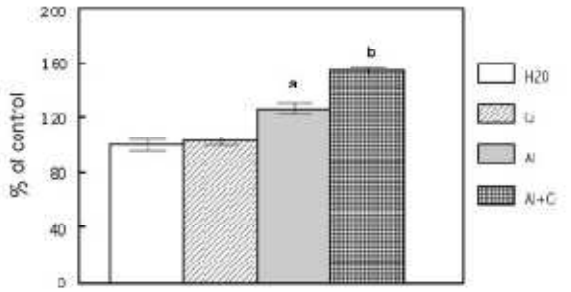

Fig. 3. Effect of oral Al administration on AChE activity in the striatum of adult rats. Each column represents mean ± SD ($n = 6$) as percent of control. AChE control value was 17.1 ± 3.7 and was expressed as μmol AcSCh/h/mg of protein. [a] Different from the others when $p < 0.01$ (ANOVA – Tukey–Kramer's test). [b] Different from Al group when $p < 0.01$ (ANOVA – Tukey–Kramer's test).

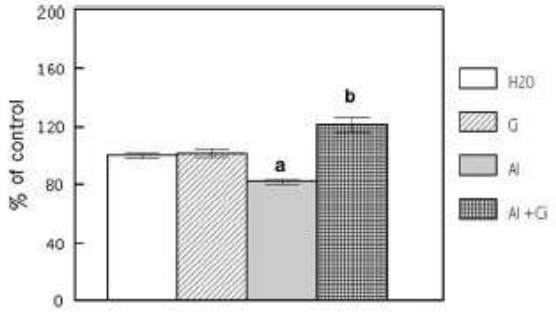

Fig. 4. Effect of oral Al administration on AChE activity in the hypothalamus of adult rats. Each column represents mean ± SD ($n = 6$) as percent of control. AChE control value was 4.53 ± 0.2 and was expressed as μmol AcSCh/h/mg of protein. The activity was expressed as μmol AcSCh/h/mg of protein. [a] Different from the others when $p < 0.01$ (ANOVA – Tukey–Kramer's test). [b] Different from Al group when $p < 0.01$ (ANOVA – Tukey–Kramer's test).

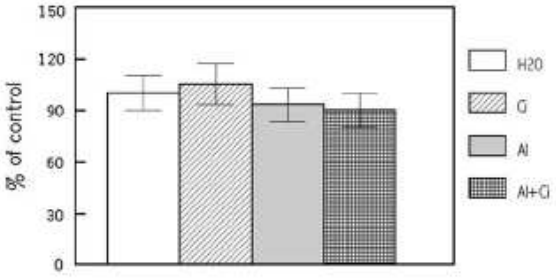

Fig. 5. Effect of oral Al administration on AChE activity in the cerebellum of adult rats. Each column represents mean ± SD ($n = 6$) as percent of control. AChE control value was 4.08 ± 1.01 and was expressed as μmol AcSCh/h/mg of protein.

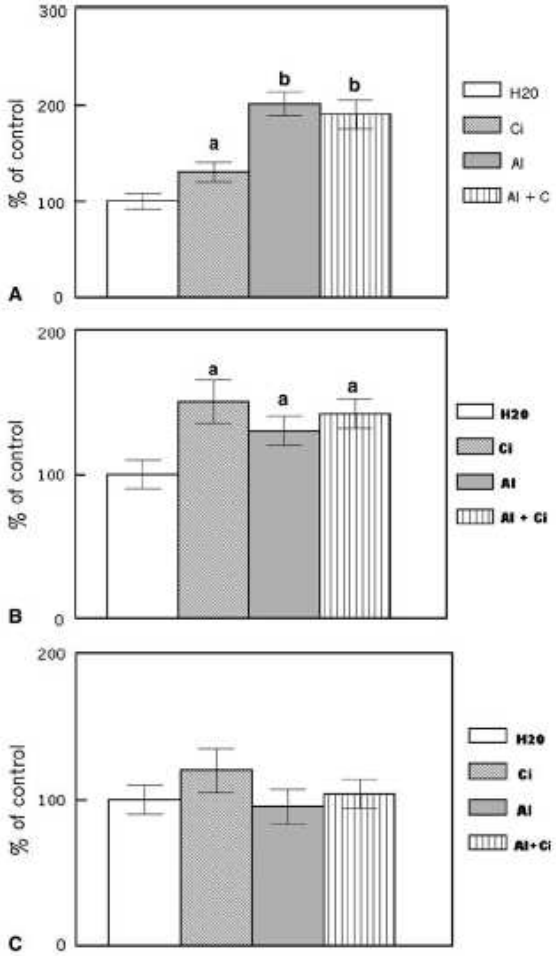

Fig. 6. Effect of oral Al administration on TBARS production in the hippocampus (A), cortex (B) and striatum (C). Each column represents mean ± SD ($n = 6$) as percent of control. The control values were 24.33 ± 3.01, 18.6 ± 3.0 and 43.3 ± 9.6 for the hippocampus, cortex and striatum, respectively. The TBARS product was expressed as nmol MDA/per mg of protein. [a]Different from the others when $p < 0.01$ (ANOVA – Tukey–Kramer's test). [b]Different from Al group when $p < 0.01$ (ANOVA – Tukey–Kramer's test).

4. Discussion

The data obtained showed that the administration of Al by gavage using this low dose caused activation of AChE activity mainly when associated with citrate (10 mg/kg) in the hippocampus, cortex and striatum. The results were not homogenous in the Al group (animals that received only Al) for the different cerebral structures studied. An elevation was observed in AChE activity in the striatum, a reduction in the hypothalamus and no alterations in the hippocampus, cortex and cerebellum. Perhaps, the different results showing both potentiating and inhibitive effects of Al on the cholinergic system are simply a reflection of the known biphasic effects of Al [34]. Another important aspect is that the hippocampus and cortex, which both receive cholinergic projections from the nucleus basalis of Meynert, presented similar results in the Al + Ci group. On the other hand, the striatum, which has an intrinsic cholinergic circuit, presented elevation in AChE activity in all Al groups. The results indicate that the effect of Al is not homogeneous in cerebral structures and that the addition of citrate always makes AChE activation more prominent. Currently, the influence of the blood brain barrier (BBB) on the pathophysiology of some neurodegenerative diseases are being discussed [35]. In fact, the pattern of brain regional sensitivity to neurotoxicants can be, in part, explained by the differences in the brain barrier mechanisms to metals [35], like Al. In this context, astrocytic foot processes are important to sequester toxic metals that have escaped the endothelial barrier.

Lipid peroxidation is an important cause of neuronal damage, as in ischaemic injuries, neurotrauma and

neurodegenerative disorders, such as Parkinson's and AD [36]. The present experiments demonstrate that Al enhanced lipid peroxides, as measured by TBARS formation, mainly in the cortex and hippocampus. A major consequence of enhanced lipid peroxidation is the oxidative deterioration of cellular membranes. Kumar [37] reported that increased Al concentration and an increased lipid peroxidation rate could affect the neurons, leading to the depletion of AChE [38]. However, here we observed that AChE was always increased, regardless of the increase in TBARS levels in brain structures. In another report [12], it was shown that long-term Al feeding altered kinetic properties of cholinesterases, presenting a reduction in the V_{max} of brain AChE in the soluble as well as membrane-bound fraction. In our experiments, we observed mainly the activation of AChE activity after a long-term exposure with a low Al oral dose. We believe that alterations in the lipid membrane could be a decisive factor in changing the conformational state of the AChE molecule. However, another explanation could be possible as reported by Gulya et al. [4], suggesting that increased AChE activity following Al exposure was due to the allosteric interaction between the cation and the peripheric anionic site of the enzyme.

Taken together, the results of TBARS indicate that Al and Al + Ci can induce oxidative stress in some specific brain regions. TBARS elevation after exposure to Al has also been reported by others [14,21,24]. In fact, Al, a non-redox-active metal is a pro-oxidant both in vivo and in vitro [22]. Al might exert its toxic effects by using mechanisms which control iron homeostasis, for example, using transport proteins such as transferrin [39]. In the brain, the choroid plexus and the oligodendrocytes are the only locations capable of producing transferrin [35]. These cells may very well be a metabolic target of Al, triggering an inability to down-regulate transferrin receptor expression and translation. In fact, even a small increase in Al in specific brain regions may be a stimulus for alterations in brain iron homeostasis [39], normally increasing Fe content, and thus contributing to neurodegenerative diseases. Taken together, this could explain the differences in lipid peroxidation in the distinct brain regions observed in this study.

In conclusion, it was observed that mainly Al + Ci altered AChE activity and enhanced lipid peroxidation, probably affecting the integrity and functionality of the cholinergic system. This shows that chronically ingested Al may be a neurotoxicant even at low doses and that the presence of citrate may exacerbate it.

Consequently, as cited by us previously [23], in order to more accurately predict the extent of human exposure to aluminum, it would be desirable to consider the level of citrate ingestion, mainly when ingested chronically.

Acknowledgments

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CAPÍTULO II

ARTIGO II:

The effect of aluminium on NTPDase and 5'-nucleotidase activities from rat synaptosomes and platelets

Rosilene Rodrigues Kaizer, Paula Acosta Maldonado, Rosélia Maria Spanevello, Maísa C. Corrêa, Jamile F. Gonçalves, Lara Vargas Becker, Vera Maria Morsch, Maria Rosa Chitolina Schetinger

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The effect of aluminium on NTPDase and 5′-nucleotidase activities from rat synaptosomes and platelets

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Abstract

Aluminium (Al), a neurotoxic compound, has been investigated in a large number of studies both *in vivo* and *in vitro*. In this study, we investigated the effect *in vivo* of long-term exposure to Al on NTPDase (nucleoside triphosphate diphosphohydrolase) and 5′-nucleotidase activities in the synaptosomes (obtained from the cerebral cortex and hippocampus) and platelets of rats. Here, we investigated a possible role of platelets as peripheral markers in rats. Rats were loaded by gavage with $AlCl_3$ 50 mg/(kg day), 5 days per week, totalizing 60 administrations. The animals were divided into four groups: (1) control (C), (2) 50 mg/kg of citrate solution (Ci), (3) 50 mg/kg of Al plus citrate (Al + Ci) solution and (4) 50 mg/kg of Al (Al). ATP hydrolysis was increased in the synaptosomes from the cerebral cortex by 42.9% for Al + Ci and 39.39% for Al, when compared to their respective control ($p < 0.05$). ADP hydrolysis was increased by 13.15% for both Al and Al + Ci, and AMP hydrolysis increased by 32.7% for Al and 27.25% for Al + Ci ($p < 0.05$). In hippocampal synaptosomes, the hydrolysis of ATP, ADP and AMP, was increased by 58.5%, 28.5% and 25.92%, respectively, for Al ($p < 0.05$) and 36.7%, 22.5% and 37.64% for Al + Ci, both when compared to their respective controls. ATP, ADP and AMP hydrolysis, in platelets, was increased by 172.3%, 188.52% and 92.1%, respectively in Al + Ci, and 317.9%, 342.8% and 177.9%, respectively, for Al, when compared to their respective controls ($p < 0.05$). Together, these results indicate that Al increases NTPDase and 5′-nucleotidase activities, in synaptosomal fractions and platelets. Thus, we suggest that platelets could be sensitive peripheral markers of Al toxicity of the central nervous system.

Keywords: Aluminium; NTPDase; 5′-Nucleotidase; Synaptosomes; Cerebral cortex; Hippocampus; Platelets

1. Introduction

Aluminium (Al) is the most abundant metal on earth; however, no connection with a useful biological function has been demonstrated. The recognition of aluminium as a neurotoxic agent has been proposed, showing that the metal may play a role in the etiology of Alzheimer's disease (AD) (Crapper et al., 1973; Xu et al., 1992), being co-related with alterations with the cholinergic system (Kaizer et al., 2005; Zatta et al., 2002).

Enhanced aluminium levels have been detected in brain areas of patients with senile plaques and neurofibrillary tangles, both of which are characteristic of AD. The hippocampus and cortex are considered AD vulnerable regions because they accumulate β-amyloid (Aβ) in mice and humans (Caccamo et al., 2005). The molecular mechanism of Al neurotoxicity has not been completely elucidated. Therefore, research about the cellular mechanisms of aluminium neurotoxicity is extremely important to elucidate the risk of specific occupational and environmental exposure (Silva et al., 2002).

Al may influence the permeability of the blood brain barrier (BBB) and alter the flux of molecules and ions inside and outside of the brain (Banks et al., 1988). It is known that homeostatic mechanisms help to maintain Ca^{2+} at low

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concentrations and it is of great importance for normal neuronal function. Evidence suggests that Al may interact with Ca^{2+} binding sites and disrupt intraneuronal Ca^{2+} homeostasis (Julka and Gill, 1996). In most of the neurodegenerative diseases, in which Al has been implicated, an excess of Ca^{2+} has been reported together with high Al levels (Garruto et al., 1984).

Moreover, aluminium can be responsible for initiating neurotoxic effects, by producing changes in the structure and function of the plasma membrane. In experimental conditions, the *in vitro* incubation of synaptosomes with $AlCl_3$ at micromolar concentrations, led to increased synaptosomal membrane rigidity, especially in the hydrophilic regions (Silva et al., 2002). A similar net fluidification effect in response to Al exposure was observed in platelets (Van Rensburg et al., 1992).

The platelet model has been used in the investigation of a number of neurodegenerative disorders, such as Alzheimer's disease (Ferrarese et al., 2000). Thus, it is important to evaluate the potential toxic effects of compounds using platelets as a model of the synaptic apparatus (Borges et al., 2004), as could be the case for Al exposure. In addition, platelets express a variety of cell surface receptors, many of which are similar in both structure and binding to their neuronal counterparts (Hourani and Cusak, 1991). The similarities between platelets and neurons may be clinically important, as a number of biochemical markers show parallel changes in the CNS and in platelets during disease states (Odagaki and Koyama, 2002; Rainesalo et al., 2003).

ATP has been defined as a fast excitatory neurotransmitter inducing some specific cellular responses (Abbracchio and Burnstock, 1998; Edwards and Gibb, 1993) and is considered to be one of the most important neurotransmitters in the purinergic system (Fredholm, 1995). Extracellular ATP is involved in a large variety of physiological and pathological functions, such as neurotransmission and neuromodulation, both in the central and peripheral nervous system (Zimmermann, 1999). Acetylcholine and ATP are co-released, where ATP acts as a co-transmitter or a modulator of the cholinergic transmission, through a dual opposite modulation, acting on facilitatory P2X or inhibitory P2Y receptors (Dahm et al., 2006; Cunha and Ribeiro, 2000). Signaling through P2 is terminated by hydrolysis of ATP by ectonucleotidases (Zimmermann, 1996).

Studies have reported that the family of ectonucleotidases such as NTPDase (nucleoside triphosphate diphosphohydrolases, apyrase, ATP diphosphohydrolase, E.C. 3.6.1.5) and 5′-nucleotidase (E.C. 3.1.3.5) participate in controlling extracellular ATP levels in the synaptic cleft and also controlling purinergic neuromodulation and neurotransmission (Zimmermann, 2001). NTPDase hydrolyzes the extracellular nucleoside tri and diphosphate in the presence of Ca^{2+} or Mg^{2+}, as it is activated by these cations (Zimmermann et al., 1998). Nucleotide hydrolysis may be impaired by Al, probably because it interferes in the homeostasis of divalent cations (Schetinger et al., 1995; Moretto et al., 2004).

Many studies have reported NTPDase activity in synaptosomes obtained from the hippocampus and cerebral cortex of rats (Battastini et al., 1991; Muller et al., 1993), such as in platelets (Pilla et al., 1996). In fact, Schetinger et al. (1995) showed a significant inhibition of NTPDase activity by Al *in vitro*, observing a competitive inhibition with the divalent cation Ca^{2+}. However, the *in vivo* effect of Al on NTPDase and 5′-nucleotidase, after a long-term exposure, have never been reported. Thus, the aim of the present study was to determine whether this treatment could affect these activities and also compare the results obtained in the central nervous system (CNS) with those obtained with platelets, as a peripheral marker.

2. Experimental procedures

2.1. Animals

Adult male Wistar rats, aged 7–8 weeks, weighing 200–300 g obtained from our breeding colony were used. Animals were maintained on a 12:12 light/dark cycle, in an air-conditioned temperature (22 ± 1 °C) colony room, with free access to water and commercial chow (Supra, Brazil). All animal procedures were approved by the Institutional Ethical Committee of the Federal University of Santa Maria (Protocol number 23/2006).

2.2. Materials

Nucleotides, Trizma Base and Percoll were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All others reagents used in the experiments were of analytical grade and of the highest purity.

2.3. Treatment

Aluminium was administered by gavage for 5 consecutive days followed by 2 days of no treatment each week, completing a total of 60 administrations *per* animal. The time of exposition to Al was determined in accordance with previous work in our laboratory (Missel et al., 2005; Kaizer et al., 2005). The dose of 50 mg/kg was selected following the literature (Julka and Gill, 1996; Silva et al., 2002; El-Demerdash, 2004).

Gavage was performed using a syringe with a modified steel point to introduce the solution into the rat's esophagus without injuring the tissue. The volume administered by gavage was related with the weigh of the animal, and it was around 400 μL. Rats were divided into four groups: (1) control, that received only ultrapure water ($n = 7$); (2) animals treated with 50 mg/kg of sodium citrate solution ($n = 7$); (3) animals treated with $AlCl_3$ 50 mg/kg plus 50 mg/kg of sodium citrate solution, ($n = 7$); (4) animals treated with $AlCl_3$ 50 mg/kg diluted in ultra pure water, ($n = 7$). The animals were euthanized 24 h after the last dose and the brains were dissected and collected immediately in beakers and maintained on ice (5 °C). The sodium citrate was used to increase the aluminium absorption (Schetinger et al., 1999; Vieira et al., 2000), probably by enhancing Al solubility (Exley, 2004). In fact, in this treatment we combined Al plus citrate, because human being is normally exposed to both in water and foods (Yokel and McNamara, 2001; Silva et al., 2002).

2.4. Synaptosome preparation

Synaptosomes were isolated essentially as described by Nagy and Delgado-Escueta (1984) using a discontinuous Percoll gradient. The cerebral cortex and hippocampus were gently homogenized in 10 volumes of an ice-cold medium (medium I) containing 320 mM sucrose, 0.1 mM EDTA and 5 mM HEPES, pH 7.5, in a motor driven Teflon-glass homogenizer and then centrifuged at 1000 × *g* for 10 min. An aliquot of 0.5 mL of the crude mitochondrial pellet was mixed with 4.0 mL of an 8.5% Percoll solution and layered into an isosmotic discontinous Percoll/sucrose gradient (10%/16%). The synaptosomes that banded at the 10/16% Percoll interface were collected with a wide-tip disposable plastic transfer pipette. The synaptosomal fraction was washed twice with an isosmotic solution consisting of 320 mM sucrose, 5.0 mM HEPES, pH 7.5, and 0.1 mM EDTA by centrifugation at 15,000 × *g* to remove the contaminating Percoll. The pellet of the second centrifugation was resuspended in an isosmotic solution to a final protein concentration of 0.5–0.8 mg/mL.

Synaptosomes were prepared fresh daily and maintained at 0–4° throughout the procedure and used for NTPDase and 5′-nucleotidase assays.

2.5. Platelet-rich plasma preparation

The platelets were prepared by the method of Pilla et al. (1996), modified by Lunkes et al. (2004). Total blood was collected by cardiac puncture and placed into a flask with 129 mM sodium citrate as anticoagulant. The total blood–citrate system was centrifuged at 160 × *g* for 40 min to remove residual blood cells. The platelet-rich plasma (PRP) was centrifuged at 1400 × *g* for 20 min and washed twice by centrifugation at 1400 × *g* with 3.5 mM HEPES isosmolar buffer containing 142 mM NaCl, 2.5 mM KCl, and 5.5 mM glucose. The washed platelets were resuspended in HEPES isosmolar buffer and adjusted to 0.4–0.45 mg of protein per milliliter.

2.6. LDH

The integrity of the synaptosome and platelet preparations was confirmed by determining the lactate dehydrogenase (LDH) activity which were obtained after synaptosome and platelet lysis with 0.1% Triton X-100 and comparing it with that of an intact preparation, using the Labtest kit (Labtest, Lagoa Santa, MG, Brasil).

2.7. Assay of NTPDase and 5′-nucleotidase activities

In synaptosomal fractions NTPDase activity was determined in a reaction medium containing 5 mM KCl, 1.5 mM $CaCl_2$, 0.1 mM EDTA, 10 mM glucose, 225 mM sucrose and 45 mM Tris–HCl buffer, pH 8.0, in a final volume of 200 μL as described in a previous study from our laboratory (Schetinger et al., 2000). Twenty microliters of the enzyme preparation (8–10 μg of protein) were added to the reaction mixture and pre-incubated for 10 min at 37 °C. The reaction was initiated by the addition of ATP or ADP to a final concentration of 1.0 mM and proceeded for 20 min. The activity of 5′-nucleotidase was determined in a reaction medium containing 10 mM $MgSO_4$ in 100 mM Tris–HCl buffer, pH 7.5, at a final volume of 200 μL, as described by Heymann et al. (1984). The reaction was initiated by the addition of AMP to a final concentration of 2.0 mM and proceeded for 20 min.

In platelets, the determination of ectonucleotidase activities was carried out using the PRP preparation according to Pilla et al. (1996). Briefly, to determine the apyrase activity, 20 μL of the PRP preparation (8–10 μg of protein) was added to the system mixture, which contained 5 mM $CaCl_2$, 100 mM NaCl, 5 mM KCl, 6 mM glucose and 50 mM tris–HCl buffer, pH 7.4. The reaction was started by the addition of 20 μL of ATP or ADP (1 mM final concentration) as substrates. For AMP hydrolysis, the 5′-nucleotidase activity was carried out as described above, except that the 5 mM $CaCl_2$ was replaced by 10 mM $MgCl_2$ and the nucleotide final concentration added was 2 mM AMP (Pilla et al., 1996). For both synaptosomes and platelets, 20 μL of the enzyme preparation (8–10 μg of protein) was added to the reaction mixture and pre-incubated for 10 min at 37 °C.

Both the reactions were stopped by the addition of 200 μL of 10% trichloroacetic acid (TCA) to provide a final concentration of 5%. After chilling on ice for 10 min, 100 μL samples were taken for assay of released inorganic phosphate (Pi) by the method of Chan et al. (1986), using malachite green as the colorimetric reagent and KH_2PO_4 as standard. Controls were carried out by adding the synaptosomal fraction after TCA addition to correct for non-enzymatic nucleotide hydrolysis. All samples were run in triplicate. Enzyme activities are reported as nmol Pi released/(min mg) of protein.

2.8. Protein determination

Protein was measured by the Coomassie Blue method according to Bradford (1976) using bovine serum albumin as standard.

2.9. Statistical analysis

Data were analyzed by analysis of variance (one-way ANOVA) followed by the Duncan multiple range test, and $p < 0.05$ was considered to represent a significant difference in the analysis. All data were expressed as mean ± S.D. All analyses were performed using the Statistica version 6.0 software.

3. Results

NTPDase and 5′-nucleotidase activities from synaptosomes and platelets were modified by aluminium treatment. In the hydrolysis of ATP in synaptosomes of the cerebral cortex, the Al group presented an increase of 39.9% and 18.7% when compared to the control and citrate group, respectively ($p < 0.05$) (Fig. 1A). The Al + Ci group was increased to 42.9% and 21.4% in comparison with the control and citrate group, respectively, and there was no difference observed between the Al + Ci and Al groups ($p < 0.05$) (Fig. 1A). ADP hydrolysis in synaptosomes of the cerebral cortex presented an increase of 13.1% in the Al group and of 13.15% in the Al + Ci group when compared to the control (Fig. 1B). 5′-Nucleotidase activity in synaptosomes of the cerebral cortex was significantly higher in the Al and Al + Ci groups (32.7% and 27.25%, respectively), when compared to the control ($p < 0.05$) (Fig. 1C).

In hippocampal synaptosomes, ATP hydrolysis was significantly higher in the Al group to 58.5%, in the Al + Ci group to 36.7%, when compared to the control ($p < 0.05$) (Fig. 1D). ADP hydrolysis was significantly enhanced in the Al group to 28.5%, and in the Al + Ci group to 22.5% when compared to the control ($p < 0.05$) (Fig. 1E). The 5′-nucleotidase activity was enhanced only in the Al group to 25.92% and 37.64% in comparison with control and citrate groups. ($p < 0.05$) (Fig. 1F).

The results for platelet ATP, ADP and AMP hydrolysis were similar. There was a significant increase in the groups that received Al plus citrate and Al ($p < 0.05$). ATP hydrolysis was significantly increased in the Al group to 317.93%, and in the Al + Ci to 172.3% when compared to the control ($p < 0.05$) (Fig. 1G). ADP hydrolysis presented an increase in the Al group of 342.82% and in the Al + Ci group of 188.25% when compared to the control ($p < 0.05$) (Fig. 1H). 5′-Nucleotidase activity was increased in the Al group to 177.9% and in the Al + Ci group to 92.1% when compared to the control ($p < 0.05$) (Fig. 1I).

The measurement of LDH indicated that at least 90% of the synaptosomes and 95% of the platelets remained intact after incubation at 37 °C (data not shown).

4. Discussion

Aluminium has been greatly studied, however, its *in vivo* effect on NTPDase and 5′-nucleotidase activities still remains unknown. In the present study, we intended to compare the effect of aluminium on NTPDase and 5′-nucleotidase activities in synaptosomal fractions of the hippocampus and cerebral cortex and in platelets of rats. Thus, we intend to compare the activities of such enzymes in different subcellular fractions, either in the central or peripheral system. Blood cells such as platelets and/or lymphocytes are ideally suited for monitoring a chemical's neurotoxic effects due to number of functions

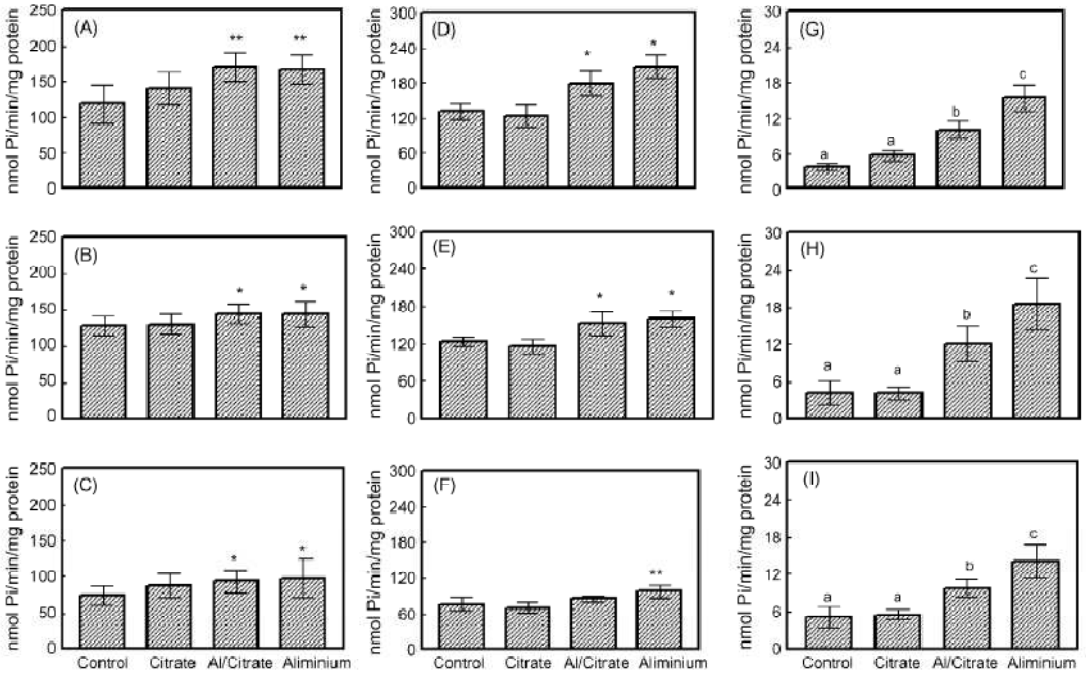

Fig. 1. ATP (A), ADP (B) and AMP (C) hydrolysis in synaptosomes of the cerebral cortex, ATP (D), ADP (E) and AMP (F) hydrolysis in synaptosomes of the hippocampus and ATP (G), ADP (H) and AMP (I) hydrolysis in platelets. *Different from the group control ($p < 0.05$). **Different from the control and citrate groups ($p < 0.05$). In platelets results, the columns not sharing the same letters are statistically different from the others ($p < 0.05$). Each column represents mean ± S.D., with $n = 7$, (ANOVA, Duncan's test).

similar to those of CNS neurons. In addition, several studies have used peripheral cells as models of nerve endings because they contain and release neurotransmitters and have receptors for neurotransmitters on their surface (Ferrarese et al., 2000; Borges et al., 2004; Odagaki and Koyama, 2002; Rainesalo et al., 2003; Chakrabarti et al., 1998).

It is known that the Al can interact with plasma membrane lipids affecting the structure and function of several proteins (Julka and Gill, 1996). We suggest that these membrane alterations can affect the activity of membrane-associated enzymes, such as NTPDase and 5′-nucleotidase.

Moreover, another question to be considered is the effect of Al on the intraneuronal Ca^{2+} homeostasis mechanism. Indeed, in most of the neurodegenerative diseases in which Al has been implicated, a Ca^{2+} excess along with Al has been reported (Shi et al., 1993). High concentrations of Al and Ca^{2+} in the CNS tissue play a critical role in neurodegeneration, producing neurofibrillary tangles and inducing cell death. Homeostatic mechanisms maintain $[Ca^{2+}]_i$ at low concentrations and are a prerequisite for normal neuronal functioning (Julka and Gill, 1996). Thus, as NTPDase and 5′-nucleotidase enzymes are activated by Ca^{+2} or Mg^{2+} cations, any alteration in the availability of ions affects the activities of these enzymes. Schetinger et al. (1995), observed a competitive inhibition between aluminium and calcium, suggesting that the aluminium ion forms complexes with ATP and ADP, since these complexes are not hydrolyzed by nucleotidases.

The present data, obtained in this study, clearly indicate that Al treatment resulted in a significant elevation of the hydrolysis of the adenine nucleotides (ATP, ADP and AMP). Besides the fact that citrate was used to enhance Al absorption, we did not observe differences between the results of the Al and Al + Ci groups. NTPDase and 5′-nucleotidase activities in platelets and synaptosomes play a pivotal role in ATP, ADP and AMP hydrolysis in these different tissues preparations (Lunkes et al., 2004). The increase in NTPDase and 5′-nucleotidase activities could be related to a compensatory response to the enhanced concentration of the intraneuronal Ca^{2+}, caused by the interference of aluminium in the ion homeostasis. This increase, also observed in NTPDase and 5′-nucleotidase activities in synaptosomal fractions of the cerebral cortex and hippocampus, demonstrates a similar cellular response in synaptosomes (CNS) and platelets, although their respective specific activities are very different. In fact, synaptosomal ectonucleotidases, which are related with neurotransmission, have higher activity than platelet ectonucleotidases, which are related with aggregation processes. Besides this, the similar patterns of changes leads us to infer that platelets could be a peripheral marker of alteration in central NTPDase and 5′-nucleotidase activities in animals exposed to Al.

In contrast to the inhibition found by Schetinger et al. (1995), using Al *in vitro*, in the present study using Al *in vivo*, an increase of NTPDase and 5′-nucleotidase activities was observed. This fact may be due to the long-term exposure to

aluminium, which could interact with the homeostasis mechanism of Ca^{2+}, therefore, increasing Ca^{2+} levels, which would facilitate the formation of nucleotide–cation complexes.

One of the major consequences of increased Ca^{2+} levels, after Al long-term exposure, is the enhanced production of free radicals, which can have detrimental effects on the integrity of cellular membranes in terms of lipid peroxidation (Julka and Gill, 1996). Perhaps, these alterations in the membrane fluidity, can affect the conformation of the enzyme, facilitating the formation of the enzyme–substrate (ES) complexes.

In conclusion, this study showed an enhanced NTPDase and 5′-nucleotidase activities both in synaptosomal fractions and platelets. Thus, these results lead us to consider that platelets can be an excellent peripheral marker of the toxicity of Al in the CNS.

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CAPÍTULO III

MANUSCRITO I:

Effect of long-term exposure to aluminium on the acetylcholinesterase activity in the central nervous system and erythrocytes

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Effect of long-term exposure to aluminium on the acetylcholinesterase activity in the central nervous system and erythrocytes

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Abstract

Aluminum (Al), a neurotoxic agent, has been associated with Alzheimer's disease (AD), which is characterized by cholinergic dysfunction in the central nervous system. In this study, we evaluated the effect of long-term exposure to aluminium on acetylcholinesterase (AChE) activity in the central nervous system in different brain regions, in synaptosomes of the cerebral cortex and in erythrocyte. The animals were loaded by gavage with $AlCl_3$ 50 mg/kg/day, 5 days per week, totalizing 60 administrations. Rats were divided into four groups: (1) control (C); (2) 50 mg/kg of citrate solution (Ci); (3) 50 mg/kg of Al plus citrate (Al+Ci), and (4) 50 mg/kg of Al (Al). AChE activity in striatum was increased by 15% for Ci, 19% for Al+Ci and 30% for Al, when compared to control ($p<0.05$). The activity in hypothalamus increased 23% for Ci, 26% for Al+Ci and 28% for Al, when compared to control ($p<0.05$). AChE activity in cerebellum, hippocampus and cortex was decreased by 11%, 23%, and 21%, respectively, for Al, when compared to their respective controls ($p<0.05$). AChE activity in synaptosomes was increased by 14% for Al, when compared to control ($p<0.05$). Erythrocyte AChE activity was increased by 17% for Al+Ci and 11% for Al, when compared to control ($p<0.05$). These results indicate that Al affects at the same way AChE activity in the central nervous system and erythrocyte. AChE activity in erythrocytes may be considered a marker of easy access of the central cholinergic status.

Keywords: Aluminium, Acetylcholinesterase (AChE), Alzheimer's Disease (AD), synaptosomes, erythrocyte.

Introduction

Metals occur naturally in the environment, but since the industrial revolution the distribution and availability of metals to biological systems have increased significantly [1]. Aluminium (Al) is one of the most abundant metal on earth; however, no connection with a useful biological function has been demonstrated [2]. It can be found in many kinds of food and medicines, such as antiacid, buffering aspirins, and antidiarrhetic [3]. In fact, evidence has shown that abnormally high Al levels are linked to important human pathologies, such as dialysis dementia, iron-adequate microcytic anemia, osteomalacia, and neurodegenerative diseases [4, 5].

The recognition of aluminium as a neurotoxic agent has been proposed, showing that the metal may play a role in the etiology of Alzheimer's Disease (AD) [6]. Al has been detected in both senile plaques and neurofibrillary tangle bearing neurons of patients with Alzheimer's disease (AD) [7], and is abundant in regions enriched for cholinergic synapses [8]. In AD the principal neurochemical abnormality is the alteration of the cholinergic system in the central nervous system (CNS) [9, 10].

Acetylcholinesterase (AChE, E.C. 3.1.1.7) is an important regulatory enzyme, which rapidly hydrolyses the neurotransmitter acetylcholine (ACh) found mainly in the brain, muscles, erythrocytes, and cholinergic neurons [11,12]. AChE activity is sensitive to exogenous factors, including diets [13] and the presence of metal such as Al [9,10]. Literature data suggest that Al interacts with the cholinergic system, acting as a cholinotoxin [14]. Its neurotoxic effects could be exerted by additional mechanisms, such as the promotion and accumulation of insoluble amyloid β protein [15]. Besides, intense AChE activity appears in the neuritic plaques and neurofibrillary tangles [16]. Alterations on the cholinergic activity are a key event in the neurochemistry of AD [17]. There is a distinct difference in AChE distribution in the cerebral cortex on histochemical studies between control subjects and

Alzheimer patients [16]. In fact, recent works of our group have investigated the effect of exogenous factors on AChE activity, such as diets [13], inhibitors [12], demyelinating model [18, 19], and the toxicity of metal, such as Al [10].

Moreover, AChE is expressed not only in the central nervous system (CNS) and peripheral nervous system (PNS), but it is also found on the surface of various blood cells. It is important to highlight that the AChE is a marker enzyme of the cholinergic activity [20]. It is also known that AD affects the cholinergic system and is reported as a systemic disorder that affects various tissues in the body [17].

In a previous work [10], we observed an increase in AChE activity of different mouse brain regions after exposure to low levels of aluminium. Thus, the aim of the present study was to determine whether a high dose (50 mg/kg) of aluminium administered by the same method (gavage) and the same time of exposition could affect the cholinergic system. Moreover, we aimed to compare the results obtained in the first supernatant with that obtained on synaptosomes and erythrocytes.

Experimental Procedures

Animals

Adult male Wistar rats, aged 7-8 weeks, weighing 200-300 g obtained from our breeding colony were used. Animals were maintained on a 12:12 light/ dark cycle, in an air-conditioned temperature (22±1ºC) colony room, with free access to water and commercial chow (Supra, Brazil). All animal procedures were approved by the Institutional Ethical Committee of the Federal University of Santa Maria (Protocol number 23/2006).

Materials

Nucleotides, Trizma Base, Percoll, acetylthiocholine iodide, and 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) were purchased from Sigma Chemical Co (St Louis, MO, USA). All other reagents used in the experiments were of analytical grade and of the highest purity.

Treatment

Aluminium was administered by gavage for five consecutive days followed by two days of no treatment each week, completing a total of 60 administrations each animal [21]. Gavage was performed using a syringe with a modified steel point to introduce the solution into the rat's esophagus without injuring the tissue. Rats were divided into four groups: (1) control, which received only ultrapure water (n=7); (2) animals treated with 50 mg/kg of citrate solution (n=7); (3) animals treated with $AlCl_3$ 50 mg/kg plus 50 mg/kg of citrate solution, (n=7); (4) animals treated with $AlCl_3$ 50 mg/kg diluted in ultra pure water, (n=7). The animals were euthanized 24h after the last dose and the brains were dissected and collected immediately in beakers and maintained on ice (5ºC). The citrate was used to increase the aluminum absorption [22], probably by enhancing Al solubility [23].

Brain Tissue Preparation

The brain was excised rapidly and the structures were isolated (striatum, hypothalamus, cerebellum, hippocampus, and cortex). The brain regions were placed in a solution of 320 mM sucrose, 0.1 mM EDTA, 5 mM Tris-HCl, pH 7.5 (medium I), at 4ºC and weighed. Then, tissues were homogenized in 10 volumes of Medium I in a motor-driven Teflon-glass homogenizer (15 strokes at 1000 rpm) and centrifuged by 10 min at 1000 x g. The supernatant was collected and used in the AChE assay.

Synaptosome Preparation

Synaptosomes were isolated essentially as described by Nagy and Delgado-Escueta (1984) [24] using a discontinuous Percoll gradient. The cerebral cortex were gently homogenized in 10 volumes of an ice-cold medium (medium I) containing 0.32 M sucrose, 0.1 mM EDTA and 5mM HEPES, pH 7.5, in a motor driven Teflon-glass homogenizer and then centrifuged at 1000 x g for 10 min. An aliquot of 0.5 mL of the crude mitochondrial pellet was mixed with 4.0 mL of an 8.5% Percoll solution and layered into an isosmotic discontinuous Percoll/sucrose gradient (10%/16%). The synaptosomes that banded at the 10/16% Percoll interface were collected with a wide-tip disposable plastic transfer pipette. The synaptosomal fraction was washed twice with an isosmotic solution consisting of 0.32 M sucrose, 5.0 mM HEPES, pH 7.5, and 0.1mM EDTA by centrifugation at 15.000 g to remove the contaminating Percoll. The pellet of the second centrifugation was resuspended in an isosmotic solution to a final protein concentration of 0.5-0.8 mg/mL. Synaptosomes were prepared fresh daily and maintained at 0-4ºC throughout the procedure and used for AChE assay.

Acetylcholinesterase assay for brain

AChE activity was assayed in the supernatant of homogenate of the different brain regions (S1) (striatum, hypothalamus, cerebellum, hippocampus, and cortex) and in synaptosomal fraction of cerebral cortex. AChE was determined according to Ellman et al. (1961) [25], modified by Rocha et al. (1993) [26]. The reaction mixture (2 mL final volume) was composed of 100 mM phosphate buffer pH 7.5, 1 mM 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB). The method is based on the formation of yellow anion, 4,4'-dithio-bis-2-nitrobenzoic measured by absorbance at 412 nm during 2 min at 25ºC. The enzyme (40-50 µg of protein) was pre-incubated for 2 min. The reaction was initiated by adding 0.8 mM

acetylthiocholine iodide. The enzyme activity was expressed in µmoles AcSCh/h/mg of protein.

Sample of blood collection

The blood was collected in vaccuntainer tubes using EDTA as anticoagulant. The samples were hemolized with phosphate buffer, pH 7.4 containing Triton X -100 (0.03%) and appropriate storage.

Determination of Erythrocyte AChE

Erythrocyte AChE activity was determined by the modification of Elmann´s et al. (1961) [25] method as described by Worek et al. (1999) [27]. Whole blood dilutions were prepared by adding 100 µL blood to 10 mL sodium/potassium phosphate buffer 0.1 mM, pH 7.4 containing Triton X -100 (0.03%). After carefully mixed, the samples were frozen immediately and kept until analysis. The hemolizate (500 µL), phosphate buffer 0.1 mM pH 7.4, DTNB 0.3 mM, and ethopropazine 0.02 mM, a selective butyrylcholinesterase (BChE) inhibitor, were pre-incubated during 10 min at 37º C. The reaction was started by the addition of substrate AcSCh 0.45 mM, and color development was measured at 436 nm.

The specific activity of erythrocyte AChE was calculated from the quotient between AChE activity and hemoglobin content, and the results were expressed as mU/µ mol Hb.

Protein determination

Protein was measured by the Coomassie blue method according to Bradford (1976) [28] using bovine serum albumin as standard.

Statistical analysis

Data were analyzed by analysis of variance (One-way ANOVA) followed by the Duncan multiple range test, and $p<0.05$ was considered to represent a significant difference in the analysis. All data were expressed as mean ± S.D. Pearson's Correlation analyses were performed between variables.

Results

AChE activity in central nervous system and in erythrocyte was modified for the aluminium treatment. AChE activity in striatum presented an increase of 15% in the Ci group, of 19% in the Al+Ci group and of 30% in the Al group, all compared to control (Fig. 1). AChE activity in hypothalamus presented an increase of 23% in the Ci group, of 26% in the Al+Ci group and of 28% in the Al group, all compared to control (Fig. 1). AChE activity in cerebellum presented a decrease of 11% and in hippocampus of 23% both in the Al group, when compared to control (Fig. 2).

AChE activity in cerebral cortex supernatant presented a decrease of 21% in the Al group, when compared to control. AChE activity in synaptosomes of the cerebral cortex presented an increase of 14% in the Al group, when compared to control (Fig. 3). In erythrocyte, AChE activity presented an increase of 17% in the Al+Ci group and 11% in the Al group, compared to control (Fig. 4). Analyzing only the AChE activity in synaptosomes obtained from cerebral cortex and in erythrocytes there was a statistically significant correlation ($r = 0.896$, $p<0.001$) (Fig. 5).

Discussion

Aluminium (Al) has been reported as a neurotoxic element, and animals exposed to Al have been suggested as animal models for Alzheimer's disease (AD) [4]. In AD, neurofibrillary degeneration is associated with a loss of the cholinergic markers choline acetyltransferase (ChAT) and AChE [29]. Moreover, high levels of Al have been detected in brain areas where the formation of senile plaques occurs [30]. Some clinical reports demonstrated an increase in aluminium absorption during aging and certain pathologies, leading to aluminium accumulation in the body [31]. The relatively high fractional aluminium absorption observed when fasted animals were given aluminium by gavage with citrate reflects several factors [32]. This reflects the increased solubility of aluminium in a citrate solution, the enhancement of Al absorption by citrate, and the negative effect of other ions (iron and calcium) and organic factors (phytate) in diet and in the gut milieu of fed animals on aluminium bioavailability [33, 34]. Although previous works of our group (10) have indicated that the addition of citrate makes AChE activation more prominent, in this present study the group treated with Al plus citrate presented no significant alteration in enzyme activity in comparison to group Al.

The effects of Al ions on biological membranes have been extensively described [35, 36]. Recently, aluminium was demonstrated to be capable not only of crossing the blood-brain barrier (BBB) [35], but also of increasing its permeability [5]. The aluminium-induced decrease of synaptic plasma membrane order is mainly associated with the reduction of cholesterol/phospholipids (CH/PL) molar ratio, rather than to an alteration of membrane phospholipid composition or direct interaction of aluminium with negatively charged membrane residues [37]. Studies have showed that the properties of membrane-bound AChE activity in the brain and the phospholipids composition of rat brain synaptic plasma membranes, microsomes and myelin as well as the Na^+, K^+ ATPase kinetics were altered

significantly under these treatment conditions [38]. AChE is an enzyme which presents similar catalytic activities but differ in hydrodynamic parameters and ionic or hydrophobic interactions [39]. In AD a selective loss of AChE molecular forms [40] occurs. In this study, the activity of the globular form of AChE in homogenized of different brain regions and synaptosomes of the cerebral cortex and erythrocytes was determined.

The data obtained in this work showed that the administration of Al by gavage caused an increase of AChE activity in the synaptosomes of the cerebral cortex and in erythrocytes. AChE activity was also enhanced in homogenized of the striatum and hypothalamus. However, AChE activity in the cerebral cortex S1 was decreased, such as in cerebellum and hippocampus. Perhaps, the different results obtained in both enhancement and inhibition of the AChE activity, in synaptosomes and S1 of cerebral cortex, may be related to the different membrane composition and the presence of different AChE molecular forms or a reflection of the biphasic effect of aluminium. Moreover, the different results found in S1 can be related with the different cholinergic projections in the structures. For example, the cerebral cortex and hippocampus received cholinergic projections from the nucleus basalis of Meynert, while the striatum has an intrinsic cholinergic circuit [10]. Therefore, the Al may interfere so differently in different brain structures.

Al can interact with plasma membrane lipids affecting the structure and function of several proteins [41]. These membrane alterations can affect the activity of membrane-associated enzymes [2]. Gulya et al. (1990) [14] have suggested that an increase in AChE activity following aluminium exposure was due to allosteric interaction between the cation in the peripheral anionic site of the enzyme molecule.

The biphasic effect of aluminium has been reported in diverse cell systems [42]. The biphasic effect of Al may be due to the formation of reversible/irreversible aluminium complex. Al has high affinity for proteins, carbohydrates and polynucleotides and may exist

as a reversible macromolecular complex of cation/polyanion [43]. Thus, the biphasic effect of Al may be related to the formation of a complex between molecules found in the supernatant and the peripheral anionic site of enzyme.

The results of the AChE-dependent β-APP induction suggests that the effect of AChE was mediated mainly by the peripheral site of the enzyme [44]. Besides, evidences reported suggest that Al may act in the neurotoxic action of amyloid beta-peptide (Aβ) [45, 46], the principal component of senile plaques, which are characteristics of AD [47].

Besides, several lines of evidences suggest that AChE may have a role in AD [44]. One of them is the association of senile plaques and neurodegeneration to brain regions rich in cholinergic transmission and the presence of AChE in senile plaques [48]. Aluminium is able to increase the activity of AChE [49] and concomitantly AChE increases Aβ aggregation [50] and promotes the generation of amyloid aggregates by accelerating the assembly of Aβ peptide into Aβ fibrils [51].

The present work was performed based on the results obtained in Kaizer et al. (2005) [10], enhancing the dose of Al (50 mg/kg) and maintaining the same method and time of exposure, by gavage during three months (60 administrations). In the present study, we used more refined tissue preparations, such as synaptosomes and erythrocytes, for the determination of acetylcholinesterase activity, which was compared in the CNS and in blood cells.

In conclusion, this study showed that the treatment with Al causes alterations in the cholinergic system in the central nervous system and erythrocytes. The results obtained presented a similar behavior of AChE activity in synaptosomes and erythrocytes. Thus, as AD is considered a systemic disorder and as blood cells are easy accessible tissues, we can suggest that erythrocytes can be used as a parameter of central cholinergic status. Additionally, the biphasic effect of aluminium was observed mainly in S1 of distinct brain

regions, which may be related to the complex formation between molecules presents in S1 and the enzyme. Comparing all the results observed in CNS with those obtained in erythrocytes, which are easily accessible tissues, we can suggest that the erythrocytes can act as a peripheral marker of central cholinergic status.

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Legends

Figure 1 – Effect of oral Al administration on AChE activity in the striatum and hypothalamus of adult rats. Each column represents mean ± S.D. (n=7) as percent of control. AChE control value for striatum and hypothalamus were 20.55 ± 2.4 and 4.83 ± 0.52, respectively, and were expressed as µmol AcSCh/h/mg of protein. * Different from the group control ($p<0.05$). ** Different from the control and Ci groups ($p<0.05$). ANOVA – Duncan's Test.

Figure 2 - Effect of oral Al administration on AChE activity in the cerebellum and hippocampus of adult rats. Each column represents mean ± S.D. (n=7) as percent of control. AChE control value for cerebellum and hippocampus were 3.9 ± 0.42 and 6.13 ± 0.38, respectively, and were expressed as µmol AcSCh/h/mg of protein. ** Different from the control and Al+Ci groups ($p<0.05$). *** Different from all the others groups ($p<0.05$). ANOVA – Duncan's Test.

Figure 3 - Effect of oral Al administration on AChE activity in the supernatant and synaptosomes obtained from the cerebral cortex of adult rats. Each column represents mean ± S.D. (n=7) as percent of control. AChE control value for homogenized of the cerebral cortex was 9.91 ± 1.7 and for synaptosomes of the cerebral cortex was 7.36 ± 0.72, and were expressed as µmol AcSCh/h/mg of protein. ** Different from the control and Ci groups ($p<0.05$). *** Different from all the others groups ($p<0.05$). ANOVA – Duncan's Test.

Figure 4 - Effect of oral Al administration on erythrocyte AChE activity of adult rats. Each column represents mean ± S.D. (n=7) as percent of control. AChE control value for erythrocyte was 194.92 ± 17.2, and were expressed as mU/µmol of Hb. * Different from the Ci group ($p<0.05$). ** Different from the control and Ci groups ($p<0.05$). ANOVA – Duncan's Test.

Fig. 5 – Correlation between AChE activities in synaptosomes obtained from cerebral cortex and in erythrocytes ($r=0.896$, $p<0,001$). Pearson's correlation.

Figures

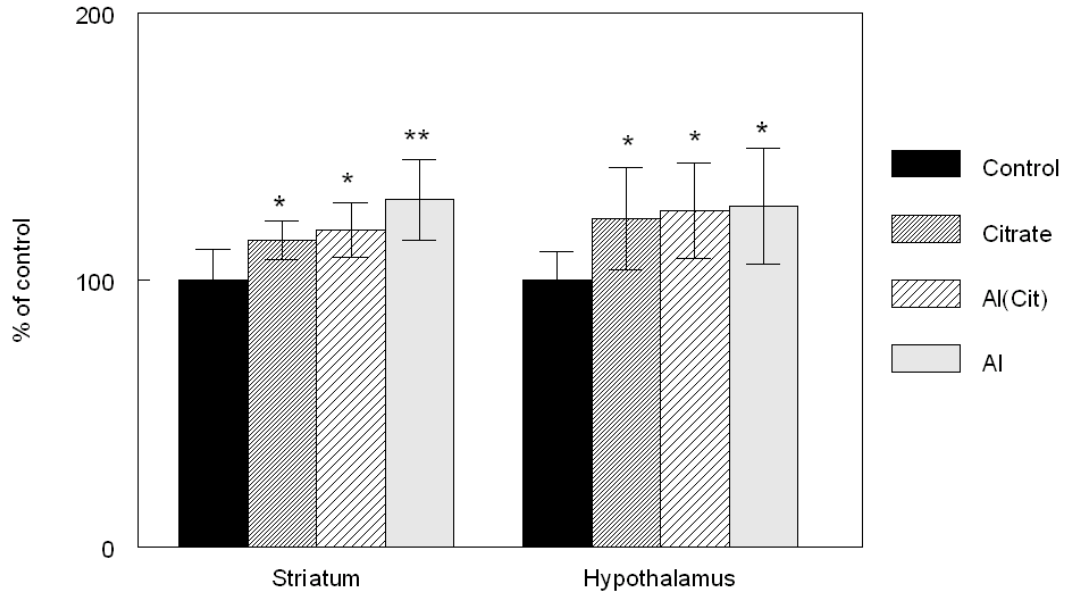

Figure 1 - AChE activity in striatum and hypothalamus.

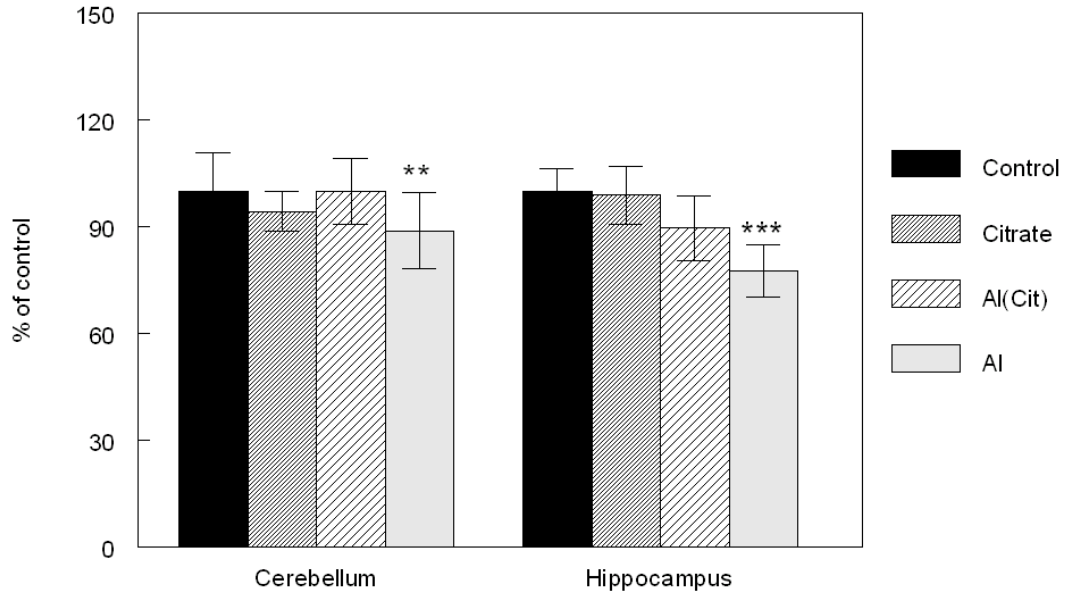

Figure 2 - AChE activity in cerebellum and hippocampus.

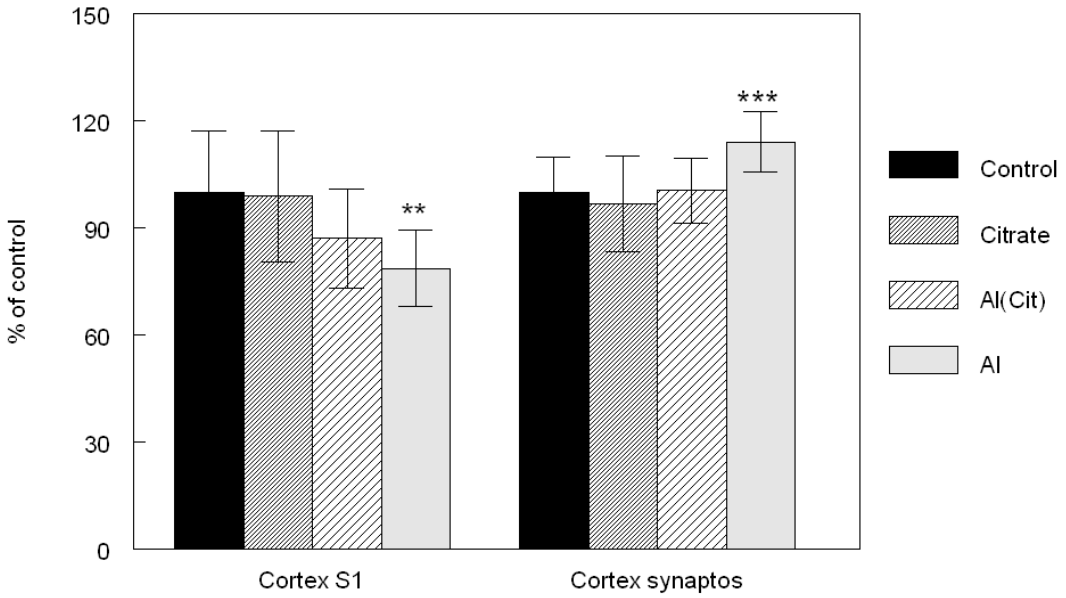

Figure 3 - AChE activity in the supernatant and synaptosomes obtained from the cerebral cortex

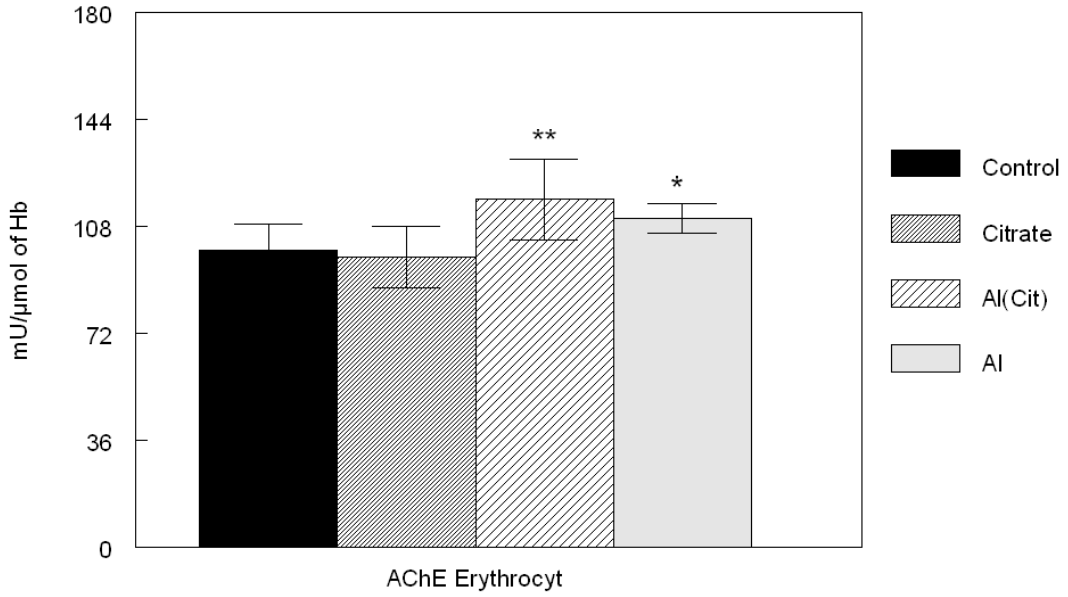

Figure 4 – Erythrocyte AChE activity.

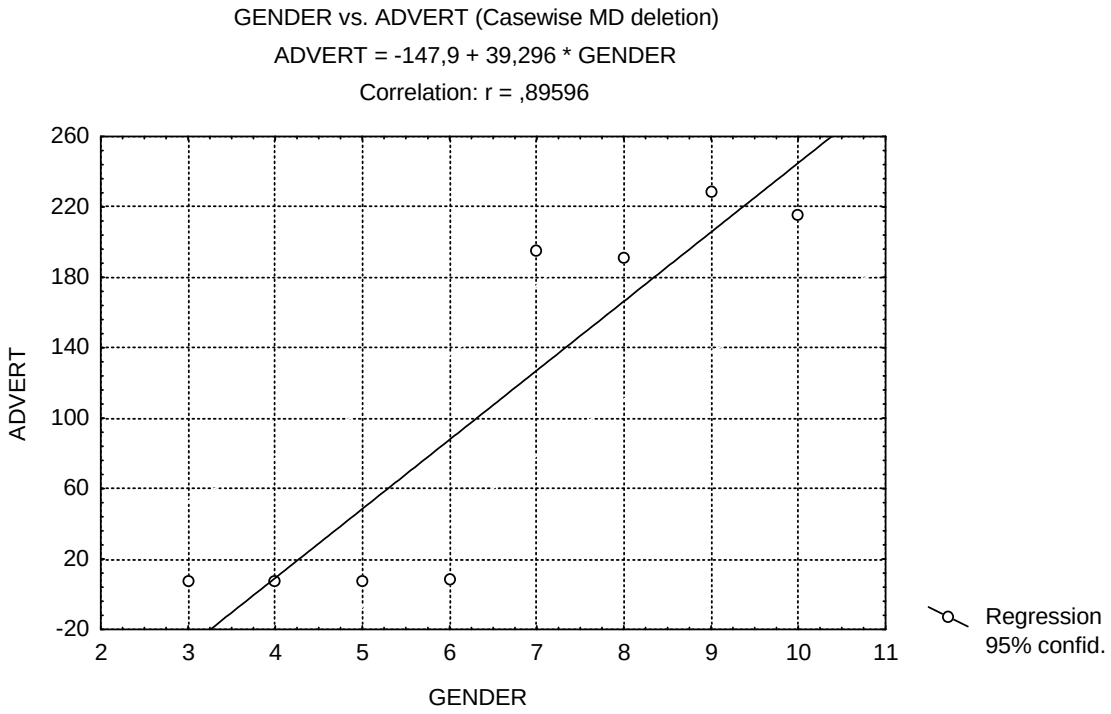

Fig. 5 – Correlation between AChE activities in synaptosomes obtained from cerebral cortex and in erythrocytes

CAPÍTULO IV

ARTIGO III:

Diet-induced changes in AChE activity after long-term exposure

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Diet-Induced Changes in AChE Activity after Long-Term Exposure

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In the present study we investigated a potential mechanism by which high sugar (HS) and high fat (HF) diets could affect acetylcholinesterase (AChE) activity. The treatment with HS and HF diet was done for six months on male and female rats. The results showed decreased hippocampal AChE activity in male and females receiving HS and HF diets (HS 24% and 36%; HF 38% and 32%, males and females, respectively; $P < 0.05$). The activity in the cerebral cortex was reduced in males (49 and 40%) and females (19 and 17%) ($P < 0.05$) on HS and HF diets, respectively. In the hypothalamus AChE activity was decreased on HS diet in males (46%) and female (25%) ($P < 0.05$) and also on HF diet in males (34%) and females (21%) ($P < 0.05$). However, in the cerebellum no changes in AChE activity were observed. These results indicate that HS and HF diets produced mainly inhibition in acetylcholine degradation. It probably indicates a chronic alteration induced by these diets on the cholinergic system.

KEY WORDS: AChE, Acetylcholine, diets, high sugar, high fat.

INTRODUCTION

It is currently well accepted that lifestyle plays a critical role in maintaining neural function in the life course of individuals. Studies mainly focus on diets typical of most industrialized Western societies, rich in saturated fat and refined sugar (HFS) (1).

The possibility that high sugar (HS) and high fat (HF) diets affect neural function would indicate that diet can increase vulnerability to numerous neurological diseases. Thus, it is important to determine the influence of dietary factors in modifying specific aspects of neuronal health and function (2).

Acetylcholine (ACh) is a neurotransmitter of pivotal importance in CNS function and is related to learning, memory, cortical organization of movement, and the control of cerebral blood flow (3). This neurotransmitter is synthesized in the nerve endings of the presynaptic nerve from choline and acetyl coenzyme A (acetyl CoA). Acetyl CoA is a product of cell metabolism, and choline is derived from lipid metabolism. After release, acetylcholine is hydrolyzed to choline and acetate by acetylcholinesterase (EC 3.1.1.7; AChE), and other non-specific esterases (4). AChE is an important regulatory enzyme that controls the transmission of nerve impulses across cholinergic synapses (5) and its activity is considered a good indicator of cholinergic activity (3).

Cholinesterases such as AChE and BuChE (butyrylcholinesterase) participate in the hydrolysis and

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inactivation of several xenobiotics, including anesthetics, cocaine, heroin, and other esters (6). Of toxicological and pharmacological significance, AChE is a target for various cholinergic toxins, such as snake venom and plant glycoalkaloids, and also a target for therapeutically active compounds, including antiAlzheimer's disease drugs and antidepressants (7,8).

In the literature there are several reports demonstrating that dietary factors affect cholinesterase activities. Inhibition of cholinesterase activities was observed in diets with varied fatty acid ratios (9), supplementation of sucrose (10), glucose (11), and plant glycoalkaloids (7).

The present work, investigate the long-term effects of HS and HF diets on the activity of AChE from the cortex, cerebellum, hypothalamus, and hippocampus. It was based on reports in the literature demonstrating that dietary factors can influence cholinesterase activities in the different conditions cited above. It is plausible to suppose that HS and HF diets can influence AChE activity. In these conditions the measurement of AChE activity could indirectly indicate the activity of the cholinergic system once synthesis and degradation of acetylcholine are a co-regulated process.

MATERIALS AND METHODS

Animals

Adult male and female Wistar rats (170–190 g) obtained from the Central Animal House Federal University of Santa Maria (Santa Maria, RS, Brazil) were housed in groups of three per cage. These animals were maintained under standard diurnal conditions (12 h light, 12 h dark), at a constant temperature of 22 ± 2 °C with *ad libitum* water and food. All animal procedures were approved by the Institutional Commission of the Federal University of Santa Maria and were in agreement with the International Council.

Materials

Acetylthiocholine iodide, 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB), thiobarbituric acid (TBA), malondialdehyde (MDA), phenylmethylsulfonyl fluoride (PMSF) and Trizma base were purchased from Sigma Chemical Co (St. Louis, MO). All other reagents used in the experiments were of analytical grade and of the highest purity.

Treatment

The animals were separated into three different groups. The (HS) diet was mostly complex carbohydrates (starch) 20% refined-sugar, 44% starch, 20% protein, 4.5% corn oil, 4.5% fiber, and 7% vitamin and mineral mix (Bionate Ltda, Brazil). The high fat (HF) diet was high in saturated fat and monounsaturated fat (same amount of lard and corn oil), 18.5% refined-sugar, 20% protein, 25% corn oil, 25% lard, 4.5% fiber, and 7% vitamin and mineral mix (Bionate Ltda, Brazil). The control group consumed standard Laboratory chow (SUPRA-RS, Brazil).

The rats were fed ad libitum and were weighed monthly. After six months of treatment with the respective diets the animals were killed by decapitation.

Brain Tissue Preparation and AChE Activity Assay

The hippocampus, cortex, cerebellum, and hypothalamus were quickly removed, placed in a solution of 320 mM sucrose, 0.1 mM EDTA, 5 mM Tris-HCl, pH 7.5 (Medium I), at 4 °C and weighed. Then, tissues were homogenized in 10 volumes of Medium I in a motor-driven Teflon-glass homogenizer (15 strokes at 1000 rpm).

Acetylcholinesterase activity was determined by the method of Ellman et al. (12), modified by Rocha et al. (13). The reaction mixture (2 ml final volume) was 100 mM phosphate buffer pH (7.5), 1 mM 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB). The method is based on the formation of a yellow anion, 4,4′-dithio-bis- nitrobenzoic acid, measured by absorbance at 412 nm after 2 min at 25 °C. The enzyme (40–50 µg of protein) was pre-incubated for 2 min. The reaction was initiated by adding 200 µl of acetylthiocholine iodide to a final concentration of 0.8 mM. The enzyme activity was expressed as µmol AcSCh hydrolyzed/h/per mg of protein.

Plasma Lipids and Glucose Levels. Plasma total cholesterol, HDL cholesterol, triglycerides, and glucose were determined by commercial colorimetric kits (Labtest—Minas Gerais—Brazil). VLDL and LDL cholesterol was calculated by the difference between plasma total cholesterol and HDL cholesterol.

Protein Determination

Protein was measured by the Coomassie blue method according to Bradford (14) using bovine serum albumin as standard.

Plasma TBARS Determination. Plasma TBARS (thiobarbituric acid-reactive substances) levels were determined by the method of Jentzsch et al. (15). Briefly, plasma (200 µl) or standard (0.03 mM MDA) was mixed with 1 ml of 0.2 M *ortho*-phosphoric acid and 250 µl TBA (0.11 mM: 800 mg TBA dissolved in 500 ml of 0.1 mM NaOH). The reaction mixture was brought to 2.0 ml with distilled water and then incubated at 90 °C for 45 min in a water bath. Plasma TBARS levels are reported as nmol MDA/ml plasma.

Liver TBARS

Lipid peroxidation was estimated by the TBARS assay. Liver TBARS levels were determined by a previously described method (16). Rat livers were promptly excised after death, weighed and homogenized at a ratio of 1 g tissue to 10 ml of 10 mM Tris-HCl buffer, pH 7.4, plus 10% sodium dodecylsulfate

(SDS). The reaction mixture contained 200 µl of the sample or standard (0.03 mM MDA), 100 µl 8.1% sodium dodecylsulfate (SDS), 400 µl of 2.5 M acetic acid solution, and 750 ml µl of 0.8% TBA. The mixture was brought to 2.0 mL with distilled water and heated at 95 °C for 90 min. After centrifugation at 5000 rpm for 5 min, absorbance was measured at 532 nm. MDA was quantified using and MDA calibration curve. Liver TBARS levels are reported as nmol MDA/h per ml tissue homogenate.

Catalase Activity Determination

Catalase (CAT) activity was measured in liver homogenates by the method of Nelson and Kiesow (17). Rat livers were promptly excised after death, divided into portions, and weighed and 1:10 tissue homogenates were prepared in 1.15% KCl solution plus 1 mM PMSF. After centrifuging at 3000 rpm for 10 min at 4 °C the supernatants were used for enzymatic reaction. The reaction mixture contained 50 mM phosphate buffer, pH 7.0, 10 mM H_2O_2 and 20 µl of the supernatant. The rate of H2O2 reaction was monitored at 240 nm for 2 min at room temperature. Catalase activity was expressed as ΔE/protein per min.

Statistical Analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey–Kramer test when $P < 0.05$.

Table I. Plasma Glucose, Cholesterol, Cholesterol Ester Concentrations in Lipoprotein Fractions and Triglyceride in Rats with a High Fat and High Sugar Diet

		PLASMA LEVELS (mg/dl)	
		Males	Females
Glucose	Control	99 ± 8.16	120 ± 9.60
	HS	110 ± 2.30	*149 ± 6.92
	HF	140 ± 23.69	*148 ± 19.4
Total-C	Control	132 ± 3.39	103 ± 9.84
	HS	*178 ± 8.08	*127 ± 15.28
	HF	*180 ± 3.60	*125 ± 6.05
HDL-C	Control	55 ± 3.60	25 ± 3.84
	HS	53 ± 4.24	*18 ± 2.51
	HF	58 ± 11.22	*19 ± 1.73
LDL-C	Control	64 ± 7.87	55 ± 5.87
	HS	**83 ± 1.85	**72 ± 2.5
	HF	**83 ± 2.86	**82 ± 2.50
VLDL-C	Control	15 ± 4.78	23 ± 2.50
	HS	**41 ± 0.57	33 ± 9.32
	HF	**39 ± 13.57	22 ± 1.4
Triglycerides	Control	63 ± 9.60	118 ± 9.14
	HS	**207 ± 2.00	*165 ± 45.8
	HF	**195 ± 67.27	108 ± 6.84

Values are the mean ± SD. $n = 4$–6 observations per group. *$P < 0.05$, **$P < 0.001$. Data were analyzed by ANOVA followed by Tukey–Kramer test.

RESULTS

Lipid plasma levels from the control group, HS group, and HF are shown in Table I. Glucose levels were enhanced ($P < 0.05$) in females on both diets, HS (24%) and HF (23%), but in males it was not observed. Cholesterol was increased about 35% in males, HS and HF ($P < 0.05$), and about 20% in females HS, and HF ($P < 0.05$). HDL-C decreased in females on both diets, HS (28%) and HF (24%) ($P < 0.05$). LDL-C increased in males on HS (173.33%) and HF (160%) diets ($P < 0.001$), but was not altered in females. Triglycerides were elevated on males HS (228.6%) and HF (209%) diets ($P < 0.001$) and in females on HF (40%) and HS diets ($P < 0.05$).

The effect of HS and HF diets on the activity of AChE from the hippocampus is shown in Fig.1. HS and HF diets decreased AChE activity in males and females (HS 24 and 36%; HF 38 and 32%, males and females, respectively; $P < 0.05$). A decrease in AChE activity was also observed in the cerebral cortex of males and females on both diets (HS 49 and 19%; HF 40 and 17%, males and females, respectively; $P < 0.05$) (Fig. 2). A similar result was obtained in the hypothalamus, where HS and HF diets inhibited AChE activity in males and females (HS 46 and 25%; HF 21 and 34%, males and females, respectively; $P < 0.05$) (Fig. 3). However, no significant changes in AChE activity were found in the cerebellum (data not shown).

TBARS plasma levels were increased in males, HS (65%), HF (40%) and in females, HS (43%) and HF (20%) ($P < 0.05$) (data not shown). Liver TBARS were increased by 115% in HF males and by 60% in HS and HF females ($P < 0.05$) (data not shown). Liver catalase activity was increased in all groups studied ($P < 0.001$), with the enhancement being more pronounced in females (about 70%) than in males (about 50%) in both dietary groups (data not shown).

DISCUSSION

It is known that people and animals with diets high in saturated fat have higher levels of total cholesterol and lower levels of HDL cholesterol (1,2,18). Roberts et al. (18) observed that higher saturated and monounsaturated fat and refined sugar caused an increase in plasma triglycerides, total-cholesterol, VLDL-C, and LDL-C, and a significant increase in the LDL-C/HDL-C ratio. In the present study, in both males and females an increase was

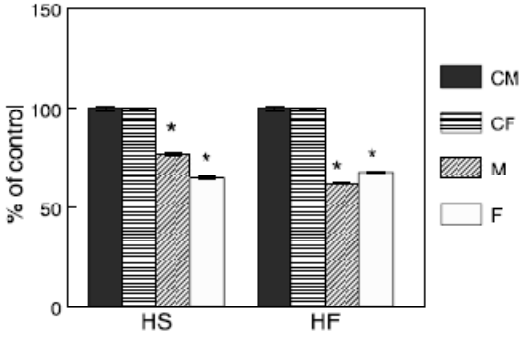

Fig. 1. Effects of HS and HF diet on AChE activity in the hippocampus. Bars represent the mean ± SD of five animals ($n = 5$). The control specific activity that represents 100% in males (7.12 ± 1.00) and in females (7.45 ± 0.17) is expressed as μmol ACSCh/h/per mg of protein. The data were analyzed by ANOVA followed by Tukey–Kramer test, *P < 0.05. Groups are described as: control males (CM); control females (CF); males (M) and females (F).

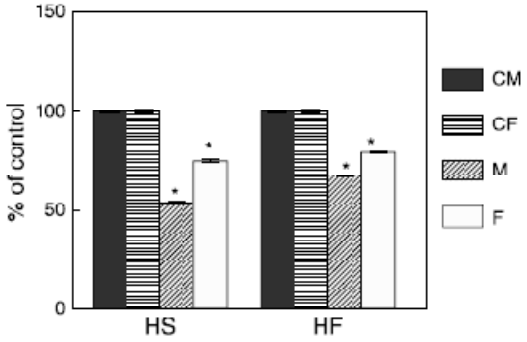

Fig. 3. Effects of HS and HF diet on AChE activity in the hypothalamus. Data represent the mean ± SD of five animals ($n = 5$). Control specific activity that represents 100% (3.37 ± 1.06) males and (3.43 ± 0.19) in females, expressed as μmol ACSCh/h/per mg of protein. The data were analyzed by ANOVA followed by Tukey–Kramer test, *P < 0.05. Groups are described as: control males (CM); control females (CF); males (M) and females (F).

also found an increase in LDL-C levels in HS and HF diets. In this way, long-term HF and HS diet can contribute to the elevation of plasma VLDL and TG. Probably the long-term enhancing in glucose, triglycerides and cholesterol levels affect the metabolism and constitution of the central nervous system and peripheral tissue, such as liver.

The diminished activity of AChE in the different structures from the central nervous system is related to HS and HF diets in both males and females. It could indicate that diet influences the activity of the cholinergic system. Reports in the literature demonstrate alteration in AChE activity

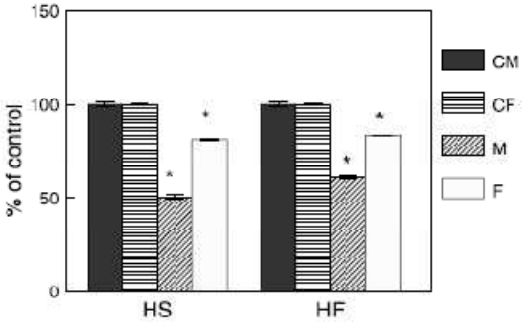

Fig. 2. Effects of HS and HF diet on AChE activity in the cerebral cortex. Data represent the mean ± SD of five animals ($n = 5$). Control specific activity represents 100% in males (7.77 ± 1.09) and in females (7.70 ± 0.15), expressed as μmol ACSCh/h/per mg of protein. The data were analyzed by ANOVA followed by Tukey–Kramer test, *P < 0.05. Groups are described as: control males (CM), control females (CF), males (M) and females (F).

with different lipid (9) and sucrose ingestion (10). There are three possibilities: first, these diets modify the microenvironment of the membrane-bound AChE. Probably alteration in cholesterol, phospholipids, and fatty acid chains occur impairing acetylcholine degradation. Second, there could be diminished synthesis of acetylcholine in both diets. Acetylcholine level was not monitored in this work, but we can speculate that the diminished hydrolysis observed is due to a regulatory mechanism that could occur in these animals. Perhaps to maintain a basal level of acetylcholine these animals modulate (decrease) the hydrolysis of this neurotransmitter. Third, diminished synthesis of the enzyme AChE could occur in both diets. The long-term treatment could cause an induction or repression of protein synthesis. Olivier et al. (11) observed that glucose feeding exacerbates parathion-induced neurotoxicity, probably because the excessive glucose consumption decreases the intake of other dietary components, in particular amino acids, limiting the '*de novo*' synthesis of AChE and the recover of synaptic transmission.

However, no changes in AChE activity were observed in the cerebellum, demonstrating that this structure was not sensitive to HS and HF diets. In fact, is acceptable that brain regions could present different vulnerability and responses in AChE activity, mainly comparing hippocampus and cerebral cortex with cerebellum. Perhaps, it could indicate the importance and vulnerability of the cholinergic via in these different brain regions.

It has been demonstrated that long-term consumption of a westernized type of diet, high in saturated fat and refined-carbohydrate, leads to oxidative stress (19). In our experiments, the hepatic lipids from males and females receiving the HF diet were more susceptible to peroxidation, demonstrating that this diet can enhance the oxidative process. On the other hand, in females the increase in lipid peroxidation did not depend on the diet and was found in both dietary groups. Probably, females are more susceptible to elevated sugar and fat levels.

Previous studies examined the relationship between oxidative stress and the effect of diet in terms of which antioxidant enzymes are altered in particular tissues. Increased antioxidant enzyme activity suggests a compensatory mechanism of defense against oxidative stress. In contrast, decreased antioxidant enzyme activity may be due to a depletion of antioxidant enzymes in response to oxidative stress. In the present study, we found an increase in liver catalase activity in both male and female rats receiving the HS and HF diets, a fact suggesting a compensatory mechanism against the oxidative stress experienced by this tissue.

In conclusion, the present results indicate that long-term HS and HF diets altered the metabolic profile of experimental animals, inhibited AChE activity in hippocampus, hypothalamus, and cerebral cortex, and enhanced lipid peroxidation and catalase activity.

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CAPÍTULO V

MANUSCRITO II:

Effect of long-term exposure to aluminium and high fat diet on NTPDase and 5'-nucleotidase activities from synaptosomes and platelets of rats

Rosilene Rodrigues Kaizer, Rosélia Maria Spanevello, Paula Acosta Maldonado, Jessié Martins Gutierres, Vera Maria Morsch and Maria Rosa Chitolina Schetinger

(Em fase de preparação para o periódico Neuroscience Research)

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Number of page: 20, figures: 2, table: 2.

Abstract

Aluminium (Al) is recognized as a neurotoxic agent and has been related to Alzheimer's disease (Al). High fat diets increased the fibrillogenesis, characteristic of AD. The present study evaluates the effect of long-term exposure to Al plus high fat diets, on NTPDase and 5'-nucleotidase activities in synaptosomes of cerebral cortex and platelets of newly weaned rat pups. The rat pups were loaded by gavage with $AlCl_3$ 50 mg/(kg day), 5 day per week, received chow *ad libitum*, during three months. The rats were divided into nine groups (n=5): (1) control; (2) high fat saturated diet; (3) high fat saturated diet and with $AlCl_3$ 50 mg/kg; (4) high fat saturated diet and with $AlCl_3$ 50 mg/kg plus 50 mg/kg of sodium citrate solution; (5) high fat saturated diet and with 50 mg/kg of sodium citrate solution; (6) high fat monounsaturated diet and with $AlCl_3$ 50 mg/kg; (7) high fat monounsaturated diet and with $AlCl_3$ 50 mg/kg; (8) high fat monounsaturated diet and with $AlCl_3$ 50 mg/kg plus 50 mg/kg of sodium citrate solution; (9) high fat monounsaturated diet and with 50 mg/kg of sodium citrate solution. ATP hydrolysis in synatpsomes in the 3, 4, 7 and 8 groups was enhanced 141.5%, 107.6%, 102.2% and 117.9%, respectively, when compared to the control group ($p<0.05$). ADP hydrolysis in synaptosomes in the 3, 4, 7 and 8 groups was enhanced 106.3%, 113.3%, 114.2% and 130.2%, respectively, in comparison to the control ($p<0.05$). 5'-nucleotidase activity in synaptosomes in the 3, 4, 7 and 8 groups was enhanced 117.8%, 138%, 118% and 128%, respectively, in relation to the control group ($p<0.05$). ATP hydrolysis in platelets in the 3, 4, 7 and 8 groups was enhanced 74%, 103%, 74.6% and 147%, respectively, when compared to the control group ($p<0.05$). ADP hydrolysis in the 3, 4, 7 and 8 groups was enhanced 137%, 176%, 117% and 189%, respectively, in comparison to the control ($p<0.05$). AMP hydrolysis in the 3, 4, 7 and 8 groups was enhanced 134%, 213%, 131% and 198%, respectively, in relation to the control group ($p<0.05$). Together, these results indicate that Al plus high fat diets increases NTPDase and 5'-nucleotidase activities, in synaptosomes and platelets of rats.

Keywords: Aluminium, diets, fat saturated, fat unsaturated, NTPDase, 5'-nucleotidase, pups, rats.

Introdution

Currently, is well established that lifestyle and environment play a critical role in maintaining neural function, and can be related to the etiology of various disorders in the life course of the individual. Exposure to metals occurs naturally in the environment, but since the industrial revolution the avaibility of metals and distribution to biological systems have increased significantly (Hopkin, 1989). Aluminium (Al) is the most abundant metal on earth, despite its abundance, no connection with a useful biological function has been discovered (Suwalsky et al., 2004). There are many sources of Al as food and medicines, such as antacid, buffering aspirins, and antidiarrhetic, is the primary route of aluminium exposure in most humans (Sutherland and Greger, 1998). Aluminium has been recognized as a neurotoxic agent that can participate in the neurotoxic action of amyloid beta-peptide (Aβ) the principal component of senile plaques, implicated in the etiology of Alzheimer's disease (Xu et al., 1992, Bondy et al., 1998; Exely and Korchazhkina, 2001). Abnormally high Al^{+3} levels are found in brain areas of patients with both senile plaques and neurofibrillary tangles, characteristic of AD (Kaizer et al., 2007).

Other factor of the blooming of industrialized western societies was the consumption of a typical diet rich in saturated fat and refined sugar. It was established that high sugar and high fat diets can contribute to cognitive decline in aging (Knopman et al., 2001) and can accelerate the course of dementia in AD (Kalmijn et al., 1997; 2000). In fact, the dietary fats are known to alter the structural membrane lipid composition, affecting the membrane-bound enzyme activities. Alteration of the cholesterol/phospholipids (CH/PL) molar ratio at the level of biomembranes may condition the vulnerability to aluminium intoxication (Silva et al., 2002). Moreover, Al can too interact with plasma membrane lipids, affecting the structure and function of several proteins (Julka and Gill, 1996; Kaizer et al., 2007). In previous work

(Kaizer et al., 2007), we observed that membrane alterations can affect the activity of membrane-associated enzymes, such as NTPDase and 5'-nucleotidase.

NTPDase (E.C. 3.6.1.5) and 5'-nucleotidase (E.C. 3.1.3.5) participate in the control of extracellular ATP levels in the synaptic cleft and in the control of purinergic neuromodulation and neurotransmission (Schetinger et al., 2001; Balz et al., 2003). NTPDase hydrolyzes the extracellular nucleosides tri and di-phosphates in the presence of Ca^{2+} or Mg^{2+} and has been well characterized in the central nervous system (CNS), in platelets and in other tissues (Pilla et al., 1996; Schetinger et al., 2001; Balz et al., 2003). A role for NTPDase in control of neurotransmission has been suggested and alterations in this enzyme activity appear to be associated with different brain processes like learning and memory acquisition (Zimmermann, 1998; 2001). In addition, the 5'-nucleotidase enzyme promote the hydrolysis of AMP to adenosine, an important neuromodulatory messenger (Zimmermann, 2001).

Kaizer et al. (2007) reported the NTPDase and 5'-nucleotidase activities in synaptosomes obtained from hippocampus and cerebral cortex and in platelets of rats. In fact, we observed that the treatment of long-term exposure to the Al enhanced the NTPDase and 5'-nucleotidase activities in both synaptosomal fractions and platelets. Similar results obtained when comparing synaptosomal fractions and platelets, confirmed the platelets as excellent peripheral marker of the toxicity of Al in the central nervous system (CNS) (Kaizer et al., 2007).

Studies demonstrated that ATP can control the acetylcholine release through a dual opposite modulation, acting on facilitatory P2X or inhibitory P2Y receptors (Cunha and Ribeiro, 2000). In fact, recent works of our group have investigated the effect of exogenous factors on AChE activity, such as diets (Kaizer et al., 2004), inhibitors (Ahmed et al., 2006), a demyelinating model (Mazzanti et al., 2006a, b), and the toxicity of metals, such as Al (Kaizer et al., 2005). Kaizer et al., (2005) observed an increase in AChE activity in

homogenized of different mouse brain regions after chronic exposure to low levels of aluminium (0.1mmol/kg). Besides, was investigated the influence of dietary factors on the AChE activity (Kaizer et al., 2004).

In previous works, we reported the isolated effect of the long-term exposure to the Al and the different diets in animals (Kaizer et al., 2004, 2005, 2007). Considering the results obtained, and the fundamental role of lifestyle and environment in association with a variety chronic diseases. In the present study, we proposed to associate these two factors, the chronic exposure to Al and diet applied in newly weaned rat pups. In fact, we chose to use pups to obtain a faster response to the treatment. Moreover, the animals received aluminium and the high fat saturated and monounsaturated diets. The Al (50mg/lkg) was administered by the same method (gavage) and time of exposition (three months), as in Kaizer et al. (2005; 2007). Thus, we intend to determine whether the long-term exposure to the association of Al and high fat diet, could affect the NTPDase and 5'-nucleotidase activities in synaptosomal fractions and platelets of rats.

2. Experimental procedures

2.1 Animals

Twenty-day-old Wistar rat pups weighing 60 g obtained from our breeding colony were used. The animals were maintained on a 12:12 light/dark cycle, in an air-conditioned (22 ± 1ºC) colony room, with free access to water and food, and were weighed weekly. All animal procedures were approved by the Institutional Ethical Committee of the Federal University of Santa Maria (Protocol number 23/2006).

2.2 Materials

Nucleotides, Trizma Base and Percoll were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents used in the experiments were of analytical grade and of the highest purity.

2.3 Treatment

The newly weaned rat pups were submitted to long-term exposure (three months) to Aluminium and a diet high in saturated or monounsaturated fat. Al was administered by gavage for 5 consecutive days followed by 2 days of no treatment each week, completing a total of 60 administrations per animal. The time of exposition to Al was determined in accordance with previous studies in our laboratory (Missel et al., 2005; Kaizer et al., 2005; Kaizer et al., 2007). The dose of 50 mg/kg was selected in accordance to the literature as a high dose (Julka and Gill, 1996; Silva et al., 2002; El-Demerdash, 2004). Gavage was performed using a syringe with a modified steel point to introduce the solution into the rat's esophagus without injuring the tissue. The volume administrated by gavage was in accordance with the weight of the animal, and it was initially around 30 µL. Therefore, the animals were weighed each week and the dose of $AlCl_3$ was adjusted accordingly.

The animals were given one of three different diets: the control group consumed standard Laboratory chow (Supra-RS, Brazil), and the treated groups received a diet high in saturated or monounsaturated fat (Table 1).

The animals were euthanized 24h after the last dose and the brain was dissected and placed immediately in beakers and maintained on ice (5ºC). Sodium citrate was used to increase aluminium absorption (Schetinger et al., 1999; Vieira et al., 2000), probably by enhancing Al solubility (Exley, 2004).

2.4 Synaptosome preparation

Synaptosomes were isolated essentially as described by Nagy and Delgado-Escueta (1984) using a discontinuous Percoll gradient. The cerebral cortex was gently homogenized in 10 volumes of an ice-cold medium (medium I) containing 320 mM sucrose, 0.1 mM EDTA and 5 mM HEPES, pH 7.5, in a motor driven Teflon-glass homogenizer and then centrifuged at 1000 x g for 10 min. An aliquot of 0.5 mL of the crude mitochondrial pellet was mixed with 4.0 mL of an 8.5% Percoll solution and layered into an isosmotic discontinous Percoll/sucrose gradient (10%/16%). The synaptosomes that banded at the 10/16% Percoll interface were collected with a wide-tip disposable plastic transfer pipette. The synaptosomal fraction was washed twice with an isosmotic solution consisting of 0.32 M sucrose, 5.0 mM HEPES, pH 7.5, and 0.1mM EDTA by centrifugation at 15,000 g to remove the contaminating Percoll. The pellet of the second centrifugation was resuspended in an isosmotic solution to a final protein concentration of 0.5-0.8 mg/mL. Synaptosomes were prepared fresh daily and maintained at 0º-4º throughout the procedure and used for NTPDase and 5'-nucleotidase assays.

Platelet-rich Plasma Preparation

The platelets were prepared by the method of Pilla et al. (1996), as modified by Lunkes et al. (2004). Total blood was collected by cardiac puncture and placed into a flask with 0.129 M sodium citrate as anticoagulant. The total blood-citrate system was centrifuged at 160 x g for 40 min to remove residual blood cells. The platelet-rich plasma (PRP) was centrifuged at 1400 x g for 20 min and washed twice by centrifugation at 1400 x g with 3.5 mmol/L HEPES isosmolar buffer containing 142 mmol/L NaCl, 2.5 mmol/L KCl, and 5.5 mmol/L glucose. The washed platelets were resuspended in HEPES isosmolar buffer and adjusted to 0.4 – 0.45 mg of protein per millilitre.

LDH

The integrity of the synaptosome and platelet preparations was confirmed by determining the lactate dehydrogenase (LDH) activity obtained after synaptosome and platelet lysis with 0.1 % Triton X-100 and comparing it with that of an intact preparation, using the Labtest kit.

Assay of NTPDase and 5'-nucleotidase activities

In synaptosomal fractions, NTPDase activity was determined in a reaction medium containing 5 mM KCl, 1.5 mM $CaCl_2$, 0.1 mM EDTA, 10 mM glucose, 225 mM sucrose and 45 mM Tris-HCl buffer, pH 8.0, in a final volume of 200 µL as described in a previous study from our laboratory (Schetinger et al., 2000). Twenty microlitres of the enzyme preparation (8-10 µg of protein) were added to the reaction mixture and pre-incubated for 10 min at 37ºC. The reaction was initiated by the addition of ATP or ADP at a final concentration of 1.0 mM and proceeded for 20 min. The activity of 5'-nucleotidase was determined in a reaction medium containing 10 mM $MgSO_4$ in 100 mM Tris-HCl buffer, pH 7.5, at a final volume of 200 µL, as described by Heymann (1984). The reaction was initiated by the addition of AMP at a final concentration of 2.0 mM and proceeded for 20 min.

In platelets, the determination of ectonucleotidases activities was carried out using the PRP preparation according to Pilla et al. (1996). Briefly, to determine the NTPDase activity, 20µL of the PRP preparation (8-10µg of protein) was added to the system mixture, which contained 5mM $CaCl_2$, 100mM NaCl, 5 mM KCl , 6 mM glucose and 50mM Tris-HCl buffer, pH 7.4. The reaction was started by the addition of 20µL of ATP or ADP (1mM final concentration) as substrates. For AMP hydrolysis, the 5'-nucleotidase activity was carried out as described above, except that the 5 mM $CaCl_2$ was replaced by 10mM $MgCl_2$ and the

nucleotide final concentration added was 2 mM AMP (Pilla et al., 1996). For the both synaptosomes and platelets, 20μL of the enzyme preparation (8-10μg of protein) was added to the reaction mixture and pre-incubated for 10 min at 37ºC.

Both the reactions were stopped by the addition of 200μL of 10% trichloroacetic acid (TCA) to provide a final concentration of 5%. After chilling on ice for 10 min, 100μL samples were taken for assay of released inorganic phosphate (Pi) by the method of Chan et al. (1986), using malachite green as the colorimetric reagent and KH_2PO_4 as standart. Controls were carried out by adding the synaptosomal fraction after TCA addition to correct for non-enzymatic nucleotide hydrolysis. All samples were run in triplicate. Enzyme activities are reported as nmol Pi released/(min mg) of protein.

Protein determination

Protein was measured by the Coomassie blue method according to Bradford (1976) using bovine serum albumin as standard.

Statistical analysis

Data were analyzed by analysis of variance (One-way ANOVA) followed by the Tukey-Kramer multiple comparison test, and $p<0.05$ was considered to represent a significant difference in the analysis. All data were expressed as mean ± S.D.

Results

The measurement of LDH indicated that at least 90% of the synaptosomes and 95% of the platelets remained intact after incubation at 37ºC (data not shown). Therefore, we can affirm that tissue preparations, synaptosomes and platelets used in the assays of the enzyme preserved the proprieties of the plasma membrane. NTPDase and 5'-nucleotidase activities

from synaptosomes and platelets were modified by aluminium and diet. In the hydrolysis of ATP in cerebral cortex synaptosomes, group 3 presented an increase of 141.5%, 92.9%, 74.3%, 94.4% and 74.4% when compared to groups 1, 2, 5, 6 and 9, respectively ($p<0.05$) (Fig. 1A). Group 4 showed an increase of 107.6%, 65.9%, 50%, 67% and 50% in comparison with groups 1, 2, 5, 6 and 9, respectively ($p<0.05$) (Fig. 1A). Group 7 presented an increase of 102.2%, 61.5%, 46%, 62.7% and 46% when compared to groups 1, 2, 5, 6 and 9, respectively ($p<0.05$) (Fig. 1A). Group 8 presented an increase of 117.9%, 74%, 57%, 75% and 57% when compared to groups 1, 2, 5, 6 and 9, respectively ($p<0.05$) (Fig. 1A).

ADP hydrolysis in cerebral cortex synaptosomes was increased in group 3 by 106.3%, 101.5%, 68%, 73.4% and 69.5% in comparison with groups 1, 2, 5, 6 and 9, respectively ($p<0.05$) (Fig. 1B). Group 4 presented an increase of 113.3%, 108.4%, 74%, 79.4% and 75.3% in comparison to groups 1, 2, 5, 6 and 9, respectively ($p<0.05$) (Fig. 1B). Group 7 showed an enhancement of 114.2%, 109%, 74%, 80% and 76% when compared to groups 1, 2, 5, 6 and 9, respectively ($p<0.05$) (Fig. 1B). Group 8 showed an enhancement of 130.2%, 125%, 87.6%, 93.6% and 89% when compared to groups 1, 2, 5, 6 and 9, respectively ($p<0.05$) (Fig. 1B).

5’-nucleotidase activity in cerebral cortex synaptosomes was increased in group 3 by 117.8%, 95%, 117%, 94%, 101% when compared to groups 1, 2, 5, 6 and 9, respectively ($p<0.05$) (Fig. 1C). Group 4 showed an enhancement of 138%, 113%, 137%, 112.4% and 120% in comparison with groups 1, 2, 5, 6 and 9, respectively ($p<0.05$) (Fig. 1C). Group 7 presented an increase of 118%, 95.6%, 118%, 95% and 102% when compared to groups 1, 2, 5, 6 and 9, respectively ($p<0.05$) (Fig. 1C). Group 8 showed an enhancement of 128%, 105%, 128%, 104% and 111% when compared to groups 1, 2, 5, 6 and 9, respectively ($p<0.05$) (Fig. 1C).

The results for NTPDase and 5'-nucleotidase activities in platelets were similar. There was a significant increase in the groups that received the high fat diet and Al plus citrate in comparison to the animals treated with a high fat diet and Al. ATP hydrolysis was increased in group 3 by 74%, 52%, 44%, 51% and 43% when compared to groups 1, 2, 5, 6 and 9, respectively ($p<0.05$) (Fig. 2A). Group 4 showed an increase of 103%, 77%, 68%, 75.6% and 66% in comparison to groups 1, 2, 5, 6 and 9, respectively ($p<0.05$) (Fig. 2A). Group 7 presented an increase of 74.6%, 52%, 44%, 51% and 43% when compared to groups 1, 2, 5, 6 and 9, respectively ($p<0.05$) (Fig. 2A). Group 8 presented an increase of 147%, 87%, 77%, 86% and 76% when compared to groups 1, 2, 5, 6 and 9, respectively ($p<0.05$) (Fig. 2A).

ADP hydrolysis in platelets was increased in group 3 by 137%, 104.8%, 74.7%, 104% and 80% in comparison to groups 1, 2, 5, 6 and 9, respectively ($p<0.05$) (Fig. 2B). Group 4 presented an increase of 176%, 138%, 103%, 137.6% and 109% when compared to groups 1, 2, 5, 6 and 9, respectively ($p<0.05$) (Fig. 2B). Group 7 showed an increase of 117%, 87.6%, 60%, 87% and 65% in comparison to groups 1, 2, 5, 6 and 9, respectively ($p<0.05$) (Fig. 2B). Group 8 presented an increase of 189%, 150%, 113%, 149% and 120% when compared to groups 1, 2, 5, 6 and 9, respectively ($p<0.05$). Group 8 also presented an increase of 33% in relation to group 4, ($p<0.05$) (Fig. 2B).

5'-nucleotidase activity was increased in group 3 by 134%, 90%, 74%, 83% and 71% in comparison groups 1, 2, 5, 6 and 9, respectively ($p<0.05$) (Fig. 3A). Group 4 showed an enhancement of 213%, 159%, 132%, 146% and 129% in comparison to groups 1, 2, 5, 6 and 9 group, respectively ($p<0.05$). AMP hydrolysis in group 4 was also increased by 34% and 35% in comparison to groups 3 and 7, respectively ($p<0.05$) (Fig. 3A). Group 7 showed an increase of 131%, 88%, 72%, 81% and 69% when compared to groups 1, 2, 5, 6 and 9, respectively ($p<0.05$) (Fig. 3A). Finally, group 8 showed an enhancement of 193%, 138%, 117%, 130% and 115% when compared to groups 1, 2, 5, 6 and 9 respectively ($p<0.05$).

Group 8 presented results similar to those of group 3; AMP hydrolysis was enhanced by 26% and 27% in comparison to groups 3 and 7, respectively ($p<0.05$) (Fig. 3A).

The animals were weighed throughout the treatment, and there was no statistically significant difference between groups (data not shown).

Discussion

In our previous study, an aluminium-induced enhancement in NTPDase and 5'-nucleotidase activities in rat cerebral cortex and platelet synaptosomes was demonstrated (Kaizer et al., 2007). In that study, the animals consumed standard laboratory chow concomitantly with the Al treatment, and it was found that the chow *per se* did not cause alteration in the enzyme activities. Therefore, in the present study the laboratory chow was used only for the control group.

In the present study, the data obtained showed that long-term exposure to the two exogenous factors, Al and a high fat diet, in association altered NTPDase and 5'-nucleotidase activities in both cerebral cortex synaptosome and platelets. Indeed, a similar behavior in the synaptosomal fraction and platelets was found in a previous study from our laboratory (Kaizer et al., 2007). These data corroborate with several studies that have used peripheral cells as models of nerve endings, because they contain and release neurotransmitters and have receptors for neurotransmitters on their surface (Chakrabarti et al., 1998; Ferrarese et al., 2000; Borges et al., 2004).

We clearly observed that Al and a high fat diet enhanced the hydrolysis of adenine nucleotides (ATP, ADP and AMP) in the groups exposed concomitantly to both factors. Furthermore, the administration of Al with both a saturated and monounsaturated fat diet resulted in a significant elevation of ADP and AMP hydrolysis in platelets when associated

with citrate. We combined Al with citrate because humans are normally exposed to both in water and foods (Yokel et al., 1999; Yokel et al., 2001; Silva et al., 2002).

Al may play a role in the initiation of amyloid fibril formation in Alzheimer's disease (AD). In fact, it was proposed that the interaction of both the amyloid precursor protein and soluble Aβ with ion transport systems represents an early step in impairing neuronal function, preceding plaque formation (Mark et al., 1997; Lauderback et al., 1999; Lovell et al., 1999; Kourie, 2001). Several studies have suggested that $AlCl_3$ interferes, indirectly, with site-site interactions that promote α-β, due to aluminium-induced alterations of membrane fluidity, enhancement of free radical production and impairment of the phosphorilation cascade (Exley, 1999; Yokel, 2000; Szutowicz, 2001; Silva et al., 2002), which, in turn, constitute factors that modulate the activity of the membrane-bound enzyme (Therien and Blostein, 2000; Lopina, 2000; Boldyrev, 2001). In fact, the alterations observed in NTPDase and 5'-nucleotidase activities may be due to membrane alterations.

In addition, the high fat diet is another environmental factor that can lead to changes similar to those characteristically associated with AD. The addition of cholesterol to the diet consistently results in increased immunoreactivity of the amyloid beta protein within neurons of the cerebral and hippocampal cortices of these animals (Sparks et al., 1994; Sparks, 1996). Silva et al. (2002) investigated the effect of cholesterol on an aluminium-induced alteration of membrane fluidity. Based on these data, we believe that the cholesterol present in the high fat diet increases the alterations induced by aluminium. In fact, we found that Al treatment in association with a high fat diet resulted in a significant alteration of the NTPDase and 5'-nucleotidase activities.

ATP promotes the formation of thioflavinT-reactive β amyloid fibrils and also enhances this effect (Exley, 1997; Exley and Korchazhkina, 2001). Exley and Birchall (1996) suggested that the role of ATP in increasing the biological availability of aluminium was to

act as an adjuvant releasing trivalent aluminium at a controlled rate to be bound by the amyloid, and thereby to promote the formation of Thioflavin T-reactive amyloid aggregates. It was suggested that an Al-induced disruption in the extracellular signaling activity of ATP by may be involved in the etiology of Alzheimer's disease (Exley, 1999). Al is probably biologically available during the period of active brain Al homeostasis, and acting as a complex with ATP, it could exert a latent toxicity as a result of the potentiation of receptor activity involved in neurotransmission. These Al-ATP complexes were more effective in promoting fibril formation suggesting that the complexes formed are more stable with peptide (Exley and Korchazhkina, 2001).

However, Al binds to ATP in order to form a complex which is more stable than that formed with Mg (Van Rensburg et al., 1997). Thus, the formation of Al-ATP complexes in the cell cytosol (Panchalingam et al., 1991), alters the integrity of cellular membranes, which consequently can affect the conformation of the enzyme (Kaizer et al., 2007). Moreover, the ability of Al-ATP to stabilize intermediates such as enzyme-substrate complexes is well known (Womack and Colowick, 1979; Caspers et al., 1994). In this case, the activities of the enzymes NTPDase and 5'-nucleotidase increased significantly in response to Al plus a high fat diet. The association between Al and the cholesterol present in the high fat diet may lead to the formation of Al-ATP complexes and alteration of the plasma membrane, which in turn, can alter the conformation of the enzyme and consequently its activity (Korchazhkina et al., 1999; Exley and Korchazhkina, 2001; Silva et al., 2002).

It known that Al and diet can promote the deposition of Aβ, which is considered to be a target event in the etiology of AD. Thus, similar to the data found in our previous study (Kaizer et al., 2007), in this study we observed that the association between Al and a high fat diet enhanced NTPDase and 5'-nucleotidase activities in cerebral cortex synaptosomes and

platelets. This corroborates with the fact that cholesterol increases the vulnerability to aluminium intoxication, observed in the NTPDase and 5'-nucleotidase activities.

In conclusion, the results obtained in rat cerebral cortex synaptosomes and platelets following exposure to aluminium together with the ingestion of diets rich in fat demonstrate that the association of Al and cholesterol may alter the organization of the plasma membrane. Consequently, the association of aluminium plus cholesterol may produce alterations in NTPDase and 5'-nucleotidase activities, which be related with neurotoxic effects.

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Legends

Figure 1 A - ATP synaptosomes – NTPDase activity in cerebral cortex synaptosomes with ATP as substrate. The control value was 164.9 ± 25.52. [a]Different from groups (1), (2), (5), (6) and (9). Each column represents mean ± SD, with n=5, (one-way ANOVA – Tukey-Kramer Test) ($P<0.05$).

Figure 1 B - ADP synaptosomes – NTPDase activity in cerebral cortex synaptosomes with ADP as substrate. The control value was 69.3 ± 3.1. [a]Different from groups (1), (2), (5), (6) and (9). Each column represents mean ± SD, with n=5, (one-way ANOVA – Tukey-Kramer Test) ($P<0.05$).

Figure 1 C - AMP synaptosomes – 5'-nucleotidase activity in cerebral cortex synaptosomes with AMP as substrate. The control value was 64.6 ±. 5.9. . [a]Different from groups (1), (2), (5), (6) and (9). Each column represents mean ± SD, with n=5, (one-way ANOVA – Tukey-Kramer Test) ($P<0.05$).

Figure 2 A - ATP of platelets – NTPDase activity in platelets with ATP as substrate. The control value was 10.9 ± 2.4. [a]Different from groups (1), (2), (5), (6) and (9). Each column represents mean ± SD, with n=5, (one-way ANOVA – Tukey-Kramer Test) ($P<0.05$).

Figure 2 B - ADP of platelets – NTPDase activity in platelets with ADP as substrate. The control value was 5.01 ± 1.05. [a]Different from groups (1), (2), (5), (6) and (9). [b] Different from the group (3). [c] Different from the HFS diet and group (4). [d] Different from group (7). [e] Different from group (8). Each column represents mean ± SD, with n=5, (one-way ANOVA – Tukey-Kramer Test) ($P<0.05$).

Figure 2 C - AMP of platelets – 5'-nucleotidase activity in platelets with AMP as substrate. The control value was 5.5 ± 1.05. [a]Different from groups (1), (2), (5), (6) and (9). [b] Different from group (3). [c] Different from group (4). [d] Different from group (7). [e] Different from group (8). Each column represents mean ± SD, with n=5, (one-way ANOVA – Tukey-Kramer Test) ($P<0.05$).

Figure

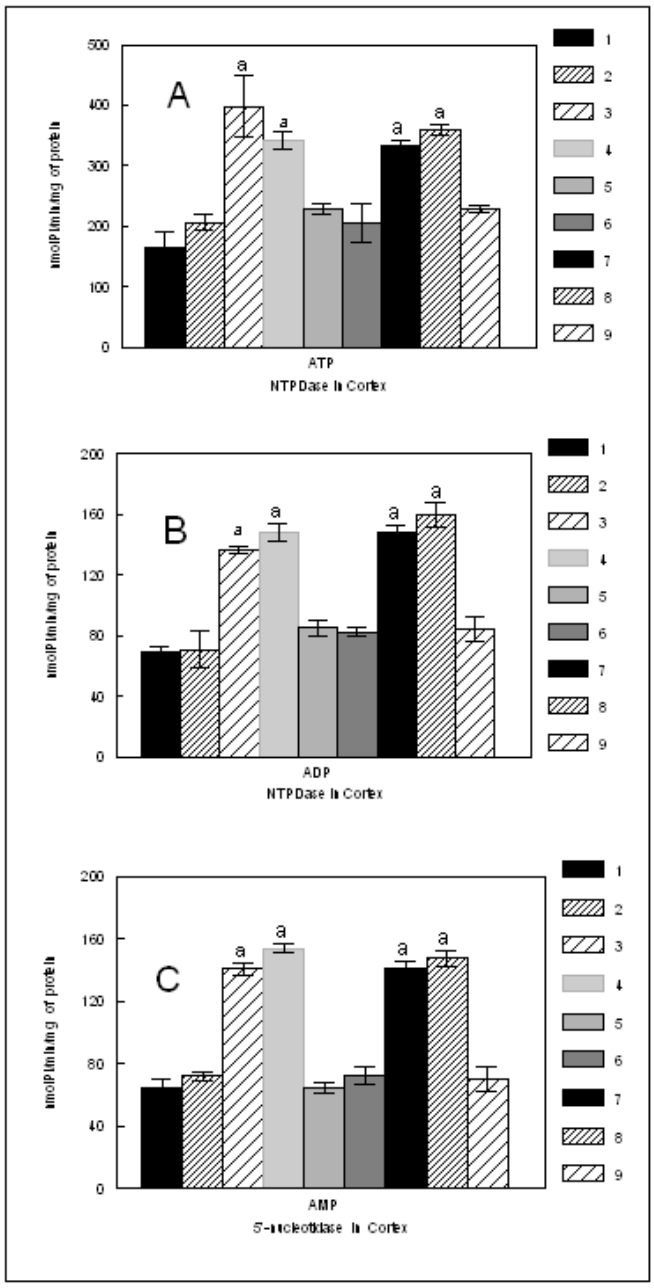

Figure 1 – ATP (A), ADP (B) and AMP (C) hydrolysis in cerebral cortex synaptosomes.

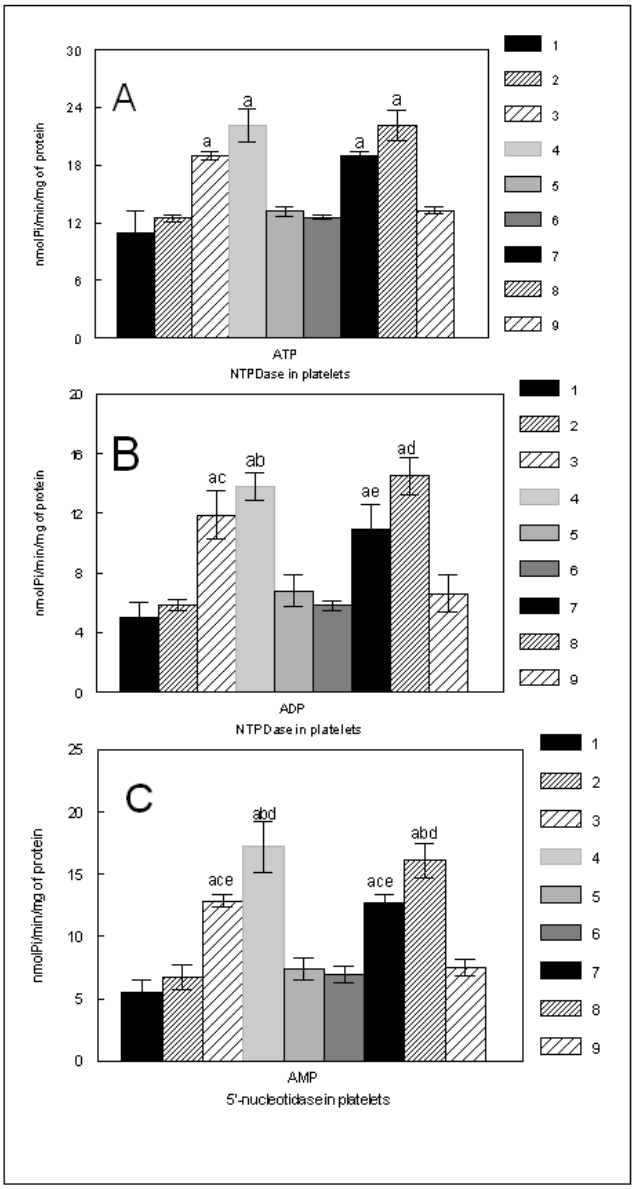

Figure 2 - ATP (A), ADP (B) and AMP (C) hydrolysis in platelets.

	High Fat Saturated Diet	High Fat Monounsaturated Diet
Protein	20%	20%
Refined-sugar	18.5%	18.5%
Lard	50%	25%
Soybeans oil	_	25%
Fibre	4.5%	4.5%
Vitamin/Mineral	7%	7%
Kcal/100g	630	630

Table 1: Diet composition

Group	Definition
1	Control - Only ultra pure water and laboratory chow
2	High fat saturated diet
3	High fat saturated diet + $AlCl_3$ 50 mg/kg diluted in ultra pure water
4	High fat saturated diet + $AlCl_3$ 50 mg/kg + 50 mg/kg of sodium citrate solution
5	High fat saturated diet + 50 mg/kg of sodium citrate solution
6	High fat monounsaturated diet
7	High fat monounsaturated diet + $AlCl_3$ 50 mg/kg diluted in ultra pure water
8	High fat monounsaturated diet + $AlCl_3$ 50 mg/kg + 50 mg/kg of sodium citrate solution
9	High fat monounsaturated diet + 50 mg/kg of sodium citrate solution

Table 2: The pups were divided into nine groups (n = 5).

4. DISCUSSÃO

O Al é considerado um agente neurotóxico (BOLLA et al., 1992; YOKEL et al., 2000), e está relacionado com a etiologia de desordens neurodegenerativas tais como encefalopatia de diálise e AD (EXLEY, 1999; EXLEY & KORCHAZHKINA, 2001; FLATEN, 2001). Além disso, o Al é considerado uma colinotoxina e um agente pró-oxidante, e seus efeitos neurotóxicos podem ser exercidos por mecanismos adicionais como a promoção e acumulação de fibrilas amilóides, além de alterações nos mecanismos de regulação do cálcio (NAYAK, 2002). Assim, este estudo foi conduzido com o propósito de avaliar possíveis alterações ocasionadas pelo Al na atividade da enzima AChE. Nesse estudo, foi determinada a atividade da AChE em homogeneizado de diferentes estruturas cerebrais de camundongos expostos por um longo período a baixa concentração de Al (0,1 mmol/kg). A dose de Al administrada foi considerada baixa, em relação a outros estudos que utilizaram a administração oral ao Al (SILVA et al., 2002; ZATTA et al., 2002; El-DEMERDASH, 2004; KOHILA et al., 2004; YOUSEF, 2004; KANEKO et al., 2004). Porém, o interesse foi desenvolver um estudo que permitisse determinar se mesmo em baixas concentrações o Al afetaria a atividade da AChE e geraria estresse oxidativo. De fato, os resultados obtidos confirmaram que mesmo em baixa dose, o Al causa alterações na atividade da AChE e peroxidação lipídica. Yokel e colaboradores (1999) relatam que o citrato de sódio aumenta a solubilidade do Al, através da formação de um complexo Al-citrato, que possibilita o influxo do Al para o encéfalo, cruzando a barreira hemato-encefálica (BBB). Com o intuito de aumentar a absorção do Al, foi administrado citrato de sódio conjuntamente ao metal. Com isso, observou-se um aumento na atividade da AChE em hipocampo, córtex, estriado e hipotálamo no grupo que recebeu Al associado ao citrato de sódio. Porém, os camundongos que receberam apenas Al, apresentaram uma inibição da atividade da AChE em hipotálamo e uma ativação em estriado, não ocorrendo alterações nas demais estruturas cerebrais. Esses resultados conflitantes, ou seja, a ativação e inibição do Al no sistema colinérgico, pode ser explicado pelo efeito bifásico do Al. Kumar (1998) afirma que o Al pode produzir um efeito bifásico, que consiste na observação de um aumento ou diminuição de qualquer resposta biológica, com o aumento da concentração de um composto teste.

Os resultados obtidos com a administração de baixas doses de Al foi um incentivo a realizar mais estudos. Na sequência, foram utilizados os mesmos grupos do trabalho anterior, com administração conjunta de Al e citrato de sódio, pelo mesmo tempo de exposição (3 meses) e método de administração, através de gavagem. Porém com algumas alterações, os animais utilizados foram ratos Wistar machos. Além disso, também foi alterada a dose para

50 mg/kg/dia de Al, seguindo dados da literatura (JULKA & GILL, 1996, SILVA et al., 2002, El-DEMERDASH, 2004). Ao mesmo tempo, utilizaram-se para a realização dos ensaios enzimáticos, preparações teciduais mais refinadas, como as frações sinaptossomais de córtex cerebral e hipocampo e preparação de plasma rico em plaquetas. Devido as alterações causadas pelo Al na atividade da AChE, e considerando que a ACh e o ATP são co-liberados na fenda sinátpica, foi investigado o efeito do Al sobre a atividade das enzimas NTPDase e 5'-nucleotidase. A determinação enzimática em plaquetas foi proposta para comparar a atividade das enzimas em um tecido de fácil obtenção, como o sangue. A exposição crônica de animais ao alumínio aumentou a hidrólise do ATP, ADP e AMP em sinaptossomas de córtex cerebral e hipocampo. A hidrólise dos nucleotídeos de purina também foi aumentada em plaquetas após exposição ao Al. O aumento da atividade de ambas as enzimas NTPDase e 5'-nucleotidase, pode estar relacionado com possíveis alterações na estrutura da membrana à qual as enzimas estão associadas, o que acarretaria numa mudança na conformação da enzima. Além disso, deve-se considerar o fato de que o Al interfere no mecanismo de homeostase intraneuronal do Ca^{2+}, níveis altos de Al são encontrados em áreas cerebrais de pacientes com AD (SILVA et al., 2003), juntamente ao Al há um excesso de Ca^{2+} (GARRUTO et al., 1984).

Como visto anteriormente, o tratamento com baixas doses de Al causou alterações na atividade da AChE. Dessa forma, o objetivo foi de investigar se o tratamento com a dose de 50 mg/kg/dia de Al, que é considerada relativamente alta, afetaria o sistema colinérgico. De fato, a exposição ao Al alterou a atividade da AChE em S1 de diferentes estruturas encefálicas, sinaptossoma de córtex cerebral e eritrócitos. A atividade da AChE em S1 foi aumentada em estriado e hipotálamo; e diminuída em cerebelo, hipocampo e córtex. Assim como no trabalho anterior, também foram encontrados resultados conflitantes entre as estruturas, dessa forma, pode-se inferir que esses resultados também estão relacionados ao efeito bifásico do Al, bem como a susceptibilidade diferenciada das áreas estudadas. Em sinaptossoma de córtex cerebral observou-se um aumento da atividade da AChE, muito semelhante ao encontrado em eritrócitos. Porém, ao contrário do aumento obtido em sinaptossoma de córtex, em S1 de córtex observou-se uma inibição da AChE. Talvez, essa diferença observada esteja relacionada ao tipo de preparação tecidual. A fração sinaptossomal de córtex é uma preparação mais refinada, utilizada em até 3 horas após sua preparação, sendo que a integridade do sinaptossoma é confirmada através da atividade da enzima lactato desidrogenase. Além disso, deve-se considerar que em S1 há grande presença de formas solúveis da AChE, enquanto em sinaptossoma a forma insolúvel da AChE é predominante.

Em adição aos resultados obtidos no SNC, a atividade da AChE em eritrócitos também foi aumentada após exposição ao Al, resultado muito semelhante ao obtido em sinaptossoma. Esse comportamento similar entre sinaptossoma e eritrócitos, pode ser justificado pelo fato de ambas as preparações, preservarem a ordem da membrana plasmática e alguns mecanismos, como o de homeostase intraneuronal do Ca^{2+} (JULKA & GILL, 1996; VERSTRAETEN & OTEIZA, 2002).

Outro fator que gera bastante interesse na sociedade, é o consumo de dietas ricas em gordura saturada e açúcar refinado. A administração de dietas ricas em gordura saturada e açúcar refinado por um longo período, inibiu a atividade da enzima AChE em homogeneizado de diferentes estruturas cerebrais de ratos machos e fêmeas. Esses resultados corroboram dados da literatura que relatam uma inibição na atividade da AChE ocasionada por fatores das dietas (RUANO et al., 2000; OLIVIER et al., 2002; MCGEHEE et al., 2000). Dessa forma pode-se inferir, que talvez a ingestão de lipídeo e sacarose, presentes na dieta, tenham modificado a estrutura da membrana plasmática, conseqüentemente alterando a conformação da AChE ligada à membrana. Outra possibilidade, seria uma inibição na síntese do neurotransmissor ACh, ocasionada por interferência dos fatores das dietas na disponibilidade de seus precursores no terminal sináptico, o que acarretaria uma inibição na atividade da própria AChE. Finalmente, pode-se considerar que a exposição por um longo período às dietas poderia influenciar o metabolismo, causando uma repressão na síntese da enzima.

Além de determinar o efeito da baixa dose de Al no sistema colinérgico, também foram avaliados os níveis de substâncias reativas ao ácido tiobarbitúrico (TBARS). Os resultados demonstraram que o Al aumentou a formação de TBARS em córtex e hipocampo, o que pode resultar em alterações na estrutura das membranas celulares. Assim, pode-se afirmar que mesmo em baixas concentrações o Al causa um aumento na peroxidação lipídica, que pode estar relacionada a alterações observadas no sistema colinérgico.

Ao mesmo tempo, os níveis de TBARS também foram aumentados, em plasma e fígado de ratos machos e fêmeas expostos às dietas ricas em gordura e açúcar. Neste estudo, também foi determinada a atividade da enzima antioxidante catalase. Dessa forma, pode-se afirmar que os animais que consumiram as dietas apresentaram maior susceptibilidade das membranas à peroxidação lipídica, devido ao estresse oxidativo. A atividade da catalase que atua na defesa antioxidante das células ao H_2O_2 foi aumentada, talvez devido a um mecanismo compensatório contra o estresse oxidativo. Sabendo que o Al interage com os lipídeos de membrana alterando a estrutura e função de várias proteínas (JULKA & GILL, 1996), pode-se considerar que essas alterações afetam a atividade de enzimas associadas à

membrana (THERIEN & BLOSTEIN, 2000; LOPINA, 2000; BOLDYREV, 2001), como é o caso da forma insolúvel da AChE, NTPDase e 5'-nucleotidase.

O ambiente desempenha um papel fundamental na vida dos organismos; a exposição individual aos fatores Al e dietas resultou em alterações enzimáticas e indução do estresse oxidativo. Considerando os resultados obtidos, realizou-se um estudo avaliando a administração conjunta de ambos os fatores Al e dietas, sobre a atividade das enzimas NTPDase e 5'-nucleotidase. A realização deste estudo é corroborada por relatos sobre o papel do colesterol no aumento da vulnerabilidade das membranas à toxicidade do Al (SILVA et al., 2002). Dessa forma, foi desenvolvido um protocolo no qual ratos machos recém-desmamados foram expostos ao Al em associação ao consumo de dietas ricas em gordura. Nesse tratamento, utilizou-se o mesmo tempo (3 meses) e dose de exposição ao Al (50mg/kg/dia) dos trabalhos anteriores. Ao mesmo tempo, foram utilizados ratos recém-desmamados para a obtenção de uma rápida resposta metabólica às dietas. Os dados obtidos relataram um aumento na atividade das enzimas NTPDase e 5'-nucleotidase em todas as dietas quando associadas ao Al e Al+Ci em sinaptossoma de córtex cerebral e plaquetas. Em adição, observou-se que a hidrólise de ADP e AMP em plaquetas foi significativamente maior nos grupos que receberam dietas em associação a Al+Ci em relação aos que receberam dietas + Al. Talvez esse fato esteja relacionado ao aumento na absorção do Al potencializado pelo citrato (KUMAR, 1998).

Um importante dado observado neste trabalho quanto à exposição ao Al, foi que mesmo em baixas concentrações (0,1 mmol/kg) este metal causa alterações no sistema colinérgico e induz a peroxidação lipídica. Quando se administraram sais de Al em uma concentração mais alta (50 mg/kg), foi confirmada a toxicidade do Al que se reflete nas alterações ocasionadas aos sistemas purinérgio e colinérgico. Além disso, pode-se observar que fatores das dietas, como lipídeos e sacarose, alteraram a atividade da AChE e induziram ao estresse oxidativo e à peroxidação lipídica. Esses resultados corroboram a hipótese de dano cognitivo e aumento da vulnerabilidade a desordens neurológicas, como AD (WINOCUR & GRENWOOD, 1999, KNOPMAN et al., 2001; KALMIJN et al., 1997; 2000). Finalmente, considerando as sociedades atuais e sua grande exposição a diversos fatores ambientais, foi investigado o efeito da associação do Al com dietas ricas em gorduras saturadas. Os resultados obtidos nesse estudo, realmente confirmaram o papel do colesterol, presente nas dietas, no aumento da vulnerabilidade das membranas à toxicidade do Al, contribuindo, assim, para as alterações observadas na atividade da NTPDase e 5'-nculeotidase que são enzimas ligadas à membrana.

5. CONCLUSÕES

- Baixas concentrações de Al (0,1 mmol/kg) alteram a atividade da enzima AChE em homogeneizado de diferentes estruturas encefálicas de camundongos machos. Os resultados da atividade da AChE foram diferentes entre as estruturas cerebrais, devido ao efeito bifásico do Al.

- A exposição a altas concentrações de Al (50 mg/kg) aumentou a atividade da NTPDase e 5'-nucleotidase em sinaptossoma de córtex cerebral, hipocampo, e plaquetas. O comportamento similar dos resultados obtidos em plaquetas e em frações sinaptossomais, sugere que as plaquetas podem ser consideradas um bom indicador periférico da toxicidade do Al no SNC.

- O Al aumentou a atividade da AChE em sinaptossomas de córtex cerebral e em eritrócitos de ratos. A atividade da AChE em S1 de diferentes estruturas encefálicas de ratos foi ativada em estriado e hipotálamo, e inibida em cerebelo, hipocampo e córtex. Essas diferenças comportamentais da enzima podem ser a manifestação do efeito bifásico do Al. A diferença encontrada entre a fração sinaptossomal e o S1 de córtex pode estar relacionada ao fato da preparação sinaptossomal preservar a ordem da membrana e preservar a atividade de enzimas ligadas à membrana. A correlação positiva da atividade das enzimas NTPDase e 5'-nucleotidase entre sinaptossoma e eritrócitos, demonstra que os eritrócitos podem ser considerados bom indicadores da função colinérgica central.

- O consumo de dietas ricas em gordura saturada e açúcar refinado por um longo período induziu a inibição na atividade da AChE nas diferentes estruturas encefálicas, que pode estar relacionada a uma inibição na síntese da ACh.

- Nos ratos expostos à baixa concentração de Al observou-se um aumento da peroxidação lipídica, em resposta ao estresse oxidativo gerado pelo metal. Os animais que consumiram dietas ricas em gordura saturada e açúcar refinado apresentaram um aumento da peroxidação lipídica e da atividade da enzima catalase, confirmando a geração de estresse oxidativo.

- A associação entre o Al e dietas com gorduras saturadas e poliinsaturadas aumentou a atividade das enzimas NTPDase e 5'-nucleotidase em sinaptossoma de córtex cerebral e plaquetas. Esse fato, sugere que o colesterol presente nas dietas aumentou o efeito tóxico do Al às membranas, alterando a conformação das enzimas ligadas à membrana. Os resultados obtidos em fração sinaptossomal e plaquetas, sugerem que as plaquetas podem ser consideradas um indicador do status purinérgico central.

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